Kode/Nama Rumpun Ilmu : 403/Biologi Farmasi
Bidang Fokus: Kesehatan Dan Obat

LAPORAN
PENELITIAN STRATEGIS NASIONAL INSTITUSI

PENGEMBANGAN EKSTRAK TAPAK LIMAN (*Elephantopus scaber Linn*) SEBAGAI KEMOPREFENTIF TERHADAP KANKER PAYUDARA

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Nomor : 109/SP2H/LT/DRPM/2018
Dan Surat Kontrak Pelaksanaan Penelitian Universitas Ahmad Dahlan
Nomor : SKIM-NO.KONTRAK/SKPP/III/2018

UNIVERSITAS AHMAD DAHLAN

NOVEMBER 2018

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Tahun Pelaksanaan	: Tahun ke 3 dari rencana 3 tahun
Biaya Tahun Berjalan	: Rp 65,000,000
Biaya Keseluruhan	: Rp 0

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RINGKASAN

Kanker payudara merupakan penyebab utama kematian pada wanita akibat kanker. Penatalaksanaan terapi kanker payudara telah berkembang, tetapi kematian akibat kanker masih tetap tinggi. Tanaman obat telah dikembangkan sebagai terapi adjuvant untuk kanker payudara. Herba tapak liman (*Elephantopus scaber* Linn) telah dilaporkan potensial sebagai antikanker dan terbukti memacu apoptosis pada penelitian in vitro (Listyowati & Nurkhasanah, 2014). **Tujuan umum penelitian** ini bertujuan untuk mengembangkan herba tapak liman dalam terapi kanker payudara. **Tujuan khusus penelitian** ini adalah: 1. Mengetahui potensi herba tapak liman sebagai antikanker payudara melalui efek sitotoksiknya pada turunan sel kanker payudara (T47D), 2 Mengetahu potensi herba tapak liman pada pemacuan apoptosis dengan mengkaji mekanisme molekulerny melalui ekspresi protein p53, Bcl-2, Bax, caspase-6 dan caspase-7 pada turunan sel kanker payudara T47D. 3, Mengetahui potensi penghambatan herba tapak liman terhadap perkembangan kanker payudara pada tikus yang diinduksi DMBA, 4. Mengembangkan bentuk sediaan herba tapak liman untuk mendapatkan potensi yang lebih baik. Penelitian yang akan dilakukan pada **Tahun I** meliputi uji in vitro mekanisme antikanker herba tapak liman terhadap turunan sel kanker payudara T47D, potensi antikanker diamati dengan melihat IC_{50} nya menggunakan metode MTT, sedangkan mekanisme molekuler ekspresi protein akan dilakukan dengan metode western bloth menggunakan antibody yang sesuai. Kuantifikasi ekspresi protein dilakukan dengan densitometri. Penelitian **Tahun II** bertujuan untuk melihat potensinya secara in vivo pada hewan coba yang diinduksi DMBA, parameter yang diamati adalah kondisi fisik hewan uji secara umum, volume tumor, ekspresi protein p53, Bcl-2, Bax, caspase-6 dan caspase-7 dari jaringan payudara. Penelitian **Tahun III** bertujuan untuk mengembangkan produk antikanker dari herba tapak liman. Produk antikanker akan dikembangkan sebagai tablet, parameter kualitas tablet yang diamati meliputi keseragaman bobot, waktu hancur, kekerasan dan kerapuhan. Dan untuk mengetahui potensi herba tapak liman dengan beberapa tanaman obat lain sebagai antikanker payudara melalui efek sitotoksiknya pada turunan sel kanker payudara (T47D), potensi antikanker diamati dengan melihat IC_{50} nya menggunakan metode MTT**. Luaran penelitian** yang diharapkan adalah: 2 jurnal internasional dan 1 paten tentang formula herbal antikanker payudara dari tapak liman.

Kata kunci: *Elephantopus scaber*, kanker payudara, antikanker

PRAKATA

Segala puji dan syukur penulis panjatkan bagi Alllah SWT karena hanya dengan rahmat, hidayah, dan karunia-Nya penulis berhasil menyelesaikan penulisan laporan kemajuan penelitian strategis nasional dengan judul –Pengembangan Ekstrak Tapak Liman (*Elephantopus scaber* Linn) Terhadap Kanker Payudara‖

Penelitian ini bertujuan untuk menggali kekayaan alam Indonesia dan memperkaya rujukan herbal Indonesia untuk peningkatan kesehatan .sesuai dengan kebijakan obat tradisional Nasional yang mengarahkan pemanfaatan dan pengembangan obat tradisional untuk penjagaan kesehatan, penyakit degenratif, dan immunostimulan, maka penelitian tapak liman ini diarahkan untuk menggali poteni sebagai antikanker.

Penulis berharap semoga peneltian ini bermanfaat bagi semua pihak dan kritik serta saran yang bersifat membangun sangat penulis harapkan. Amin.

Penulis

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BAB I
PENDAHULUAN

A. Latar Belakang

Kanker adalah penyebab kematian kedua terbesar setelah penyakit kardiovaskuler. Kanker payudara merupakan penyebab utama kematian pada wanita akibat kanker (Parkin et al. 2001). Penyebab pasti kanker payudara tidak diketahui. Para peneliti juga menemukan bahwa kerusakan dua gen yaitu BRCA1 dan BRCA2 dapat meningkatkan risiko wanita terkena kanker sampai 85%. Hal yang menarik, faktor genetik hanya berdampak 5-10% dari terjadinya kanker payudara dan ini menunjukkan bahwa faktor risiko lainnya memainkan peranan penting (Rumiyati et al. 2006).

Penatalaksanaan kanker payudara telah mengalami kemajuan yang sangat pesat, akan tetapi angka kematian dan angka kejadian kanker payudara masih tetap tinggi. Pengobatan kanker pada umumnya sama, yaitu salah satu atau kombinasi dari pembedahan, penyinaran (radioterapi), kemoterapi (sitostastik), peningkatan daya tahan tubuh dan pengobatan dengan hormon (Apantaku 2002). Pembedahan tidak dapat dilakukan pada kanker stadium lanjut khususnya pada sel kanker yang sudah bermetastatis. Kemoterapi dapat menjadi salah satu alternatif pengendalian penyakit kanker. Meskipun demikian, walaupun menunjukkan hasil yang baik kemoterapi memiliki efek samping dan toksisitas yang tinggi (Apantaku 2002). Kegagalan kemoterapi dapat berkaitan dengan kegagalan agen antikanker untuk mempengaruhi kematian sel secara terprogram (apoptosis) (Kaufmann & Earnshaw 2000). Resistensi sel kanker terhadap kemoterapi banyak dilaporkan, hal ini dapat disebabkan oleh overekspresi PgP di dalam sel yang mengakibatkan adanya efflux obat keluar sel. Oleh karena itu, pengembamgan agen sitotoksik baru untuk terapi kanker ini sangat diperlukan (Reddy et al. 2003).

Selain fenomena resistensi, kemoterapi kanker yang digunakan saat ini memiliki efek farmakologis yang kurang selektif, di samping membunuh sel kanker juga membunuh sel normal dan menimbulkan efek toksik bagi penderita penyakit kanker (Paul Symonds & Foweraker 2006). Hal ini mendorong ilmuwan untuk mencari senyawa baru yang lebih efektif dengan efek toksik seminimal mungkin.

Senyawa alami telah digunakan dalam terapi kanker (Newman et al. 2000). Sebanyak 50% obat – obat yang digunakan dalam pengobatan kanker diisolasi dari sumber alamiah atau yang berkaitan dengan sumber alamiah tersebut (Cragg & Newman 2005). Penemuan-penemuan tanaman obat yang menunjukkan efek farmakologis terhadap penyakit kanker, terutama yang telah mengalami uji secara ilmiah telah memberikan alternatif dalam mengatasi dan mengobati penyakit kanker.

Beberapa penelitian telah melaporkan khasiat herba tapak liman sebagai antikanker. Ekstrak tapak liman telah dilaporkan memilik efek toksik dan memacu terjadinya apoptosis pada sel kanker serviks (Listyowati & Nurkhasanah 2014; Xu et al. 2006), sel kanker payudara (Kabeer et al. 2014; Ho et al. 2011). Beberapa kandungan kimia yang telah dilaporkan potensial sebagai antitumor adalah deoxyelephantopin (Huang et al. 2010), scabertopinol, trans-caffeic acid, methyl 3,4-dicaffeoylquinate, luteolin-41-O-β-D-glucoside, trans-p-coumaric acid, indole-3-carbaldehyde, methyl trans-caffeate, luteolin-7-O- glucuronide 6"-methyl ester, and luteolin (Chang et al. 2011).

Penelitian ini bertujuan untuk mengembangkan produk antitumor dari herba tapak liman. Penelitian dimulai dari kajian mekanisme antikanker secara in vitro dilanjutkan kajian secara in vivo yang akan sangat diperlukan untuk pengembangan herbal ini dalam pelayanan kesehatan formal. Setelah mekanisme molekulernya diketahui, kajian dilanjutkan pada tahap formulasi dengan beberapa herbal lain untuk mendapatkan efek sinergis. Produk akan dikembangkan sebagai tablet.

B. Luaran Penelitian

1. Artikel pada jurnal internasional tentang mekanisme pemacuan apoptosis herbal tapak liman pada sel T47D, tahun I.
2. Artikel pada jurnal internasional tentang mekanisme kemoprefensi herbal tapak liman pada tikus yang diinduksi DMBA, tahun II.
3. Paten tentang formula herbal antitumor dari tapak liman dan uji sitotoksik herbal tapak liman dengan beberapa tanaman obat lain pada turunan sel kanker payudara T47D, tahun III

BAB II

TINJAUAN PUSTAKA

A. Kanker

Perkataan *cancer* dalam bahasa Latin yang bermaksud kepiting, telah diperkenalkan oleh Hippokrates pada abad kelima SM. Kanker merujuk kepada penyakit yang ditimbulkan karena sel-sel abnormal membagi secara terus menerus dengan tidak terkontrol dan dapat menyebar ke seluruh bagian tubuh (Kleinsmith 2006). Kanser telah menjadi penyebab kematian utama di kebanyakan negara. Setiap tahunnya lebih daripada 6 juta kematian disebabkan oleh kanker (Parkin et al. 2001).

Sel-sel kanser memiliki kecacatan dalam sistem pengawalaturan, yang berfungsi mengawalatur proliferasi yang normal dan homeostasis (Hanahan et al. 2000). Di antara ciri-ciri sel kanker yang membedakannya dari sel normal adalah kemampuannya untuk menghindar dari pengawasan sistem imun, menghindari apoptosis, berproliferasi secara berterusan dan kemampuannnya untuk menyebar ke seluruh tubuh (metastasis) (Hanahan et al. 2000; Cretney et al. 2007).

B. Kanker Payudara

Kanker payudara merupakan jenis kanker yang paling banyak diderita wanita di seluruh dunia.Sebagian besar penyakit kanker (lebih dari 95%) disebabkan karena pengaruh *epigenetik* dimana gen-gen dipengaruhi oleh berbagai faktor eksternal seperti makanan dan lingkungan. Penyakit ini terjadi umumnya dikarenakan terlambatnya penanganan dan pengobatan para penderita sehingga kanker sudah dalam stadium lanjut atau sudah sulit disembuhkan.

Kanker payudara adalah keganasan yang bermula dari sel-sel payudara. Kanker ini menyerang jaringan payudar, tumbuh di dalam kelenjar susu, saluran susu, dan jaringan lemak. Terjadinya karena ada pertumbuhan abnormal sel pada kelenjar payudara. Namun, pertumbuhan kanker payudara jauh lebih lambat dibandingkan dengan jenis kanker lainnya. Sistem getah bening adalah salah satu cara utama kanker payudara menyebar. Sel-sel kanker payudara dapat memasuki pembuluh limfe dan mulai tumbuh di kelenjar getah bening. Jika sel-sel kanker payudara telah mencapai pembuluh getah bening di ketiak (*node axilaris*), tandanya adalah pembengkakan kelenjar getah bening di ketiak. Bila ini terjadi, kemungkinan besar sel-sel kanker telah masuk ke aliran darah dan menyebar ke organ tubuh lainnya (Soebachman, 2011)

Kanker payudara terjadi akibat adanya mutasi tertentu pada DNA sel payudara. Sebagaian mutasi gen bersifat diwariskan (*genetik*). Sementara sebagian

yang lain tampak terjadi dengan sendirinya tanpa diketahui penyebab pastinya (Soebachman, 2011)

C. Sel T47D

Sel T47D merupakan *continous cell lines*. *Cell line* adalah sel yang disubkultur dari *primary cultures*, yaitu sel yang langsung berasal dari organ atau jaringan yang diperoleh dengan metode enzimatik maupun secara mekanik dan dikultur dalam kondisi hormonal yang sesuai (Doyle *and* Griffiths, 2000). Sel T47D merupakan *continous cell lines* yang dikultur dari jaringan epitel duktus payudara seorang wanita berusia 54 tahun. Sel T47D memiliki karakteristik ER (*Estrogen Reseptor*)/PR (*Progesteron Reseptor*)-positif. Secara molekular sel mengalami mutasi pada p53, sehingga sel kehilangan kontrol pada regulasi *cell cycle*nya Sel Vero pertama kali diambil dari ginjal *African Green Monkey* dewasa pada tanggal 27 Maret 1967 oleh T. Yasamura dan T. Kawalata dari Universitas Chiba, Chiba Jepang.

Sel ini dapat ditumbuhkan dengan medium penumbuh DMEM dengan *fetal bovine serum* 10% dan antibiotik bebas pada suhu 37ºC dan dapat tumbuh secara kontinyu, menempel pada dasar flask (Anonim, 2008).

Sel T47D merupakan sel kanker yang mengekspresikan reseptor estrogen atau yang biasa disebut ER positif serta mengekspresikan p53 yang telah termutasi. Pada sel ini p53 mengalami *missense mutation* pada residu 194 (dalam *zinc-binding domain* L2) sehingga p53 kehilangan fungsinya. Jika p53 tidak dapat mengikat *response element* pada DNA, maka akan mengurangi atau menghilangkan kemampuannya dalam meregulasi siklus sel dan memacu apoptosis (Schafer *et al*., 2000).

Media yang digunakan pada sel T47D adalah Roswell Park Memorial Institute (RPMI) 1640 serum. Media RPMI mengandung nutrisi yang dibutuhkan sel seperti asam amino, vitamin, garam-garam anorganik, dan glukosa. Serum mengandung hormon pertumbuhan sel, albumin merupakan protein transport, lipid diperlukan untuk pertumbuhan sel, dan mineral merupakan kofaktor ezim. Seluruh komponen dalam media RPMI tersebut berguna untuk memberikan nutrisiyang cukup pada sel untuk tetap bertahan hidup dan memperbanyak diri (Amalina 2008:14)

D. Apoptosis

Proses kematian sel secara umum dibagi menjadi dua yaitu apoptosis dan nekrosis (Gewies 2003). Apoptosis dikenali sebagai kematian sel terprogram

merupakan suatu proses fisiologi utama dalam pengaturan kematian sel terutamanya apabila sel mengalami kerusakan DNA yang tidak bisa diperbaiki. Selain itu, apoptosis berperanan pada pembentukan organ semasa embriogenesis, pengekalan homeostasis tisu dan penyingkiran sel yang terjangkit (Lawen 2003).

Apoptosis memainkan peranan penting dalam menstabilkan sistem homeostasis sel dan jaringan. Homeostasis ditentukan antara keseimbangan antara proliferasi sel dan kematian sel termasuk apoptosis. Apoptosis merupakan suatu proses yang aktif dan dikontrol secara genetik (Huerta et al. 2007). Gangguan pada proses apoptosis yang normal dapat mengakibatkan berbagai jenis penyakit. Pengaktifan apoptosis yang tidak terkontrol dapat menyumbang kepada beberapa penyakit seperti penyakit neurodegeneratif, Parkinson, Alzheimer. Kekurangan proses apoptosis juga boleh membawa kepada pembentukan tumor akibat proliferasi sel kanker yang berlebihan dibanding dengan kematian sel (Reed 2000).

Sel yang mengalami apoptosis dapat dikenali dari perubahan-perubahan morfologinya, di mana sel akan mengkerut dan kehilangan hubungan antara sel diikuti kondensasi kromatin di dalam nukleus. Proses selanjutnya adalah penguraian nukleus menjadi beberapa partikel terikat membran yang dikenali sebagai badan apoptotik. Jasad apoptotik yang terbentuk ini selanjutnya akan difagositosis oleh makrofag di sekitarnya. Proses ini menghalangi pembebasan kandungan lisis jasad apoptotik ke dalam ruang ekstra sel sehingga menghindari terjadinya inflamasi (Lawen 2003). Dalam *in vitro* proses fagositosis tidak terjadi, sebaliknya nekrosis sekunder seperti pengembangan sel dan lisis terjadi (Saraste & Pulkki 2000) .

Apoptosis merupakan mekanisme kontrol pertumbuhan sel yang melibatkan gen yang mengatur berlangsungnya siklus sel, diantaranya gen *p53, Rb,* serta keluarga *bcl2*. Pengaturan proliferasi sel baik akibat aktivitas onkogen dominan maupun inaktivasi *tumour supressor gene* berpengaruh pada siklus sel. Pada sel kanker, pengaturan molekuler dalam sel kanker berubah karena sel kanker memiliki kemampuan untuk menghasilkan sinyal pertumbuhan sendiri atupun hanya membutuhkan sedikit sinyal dari lingkungannya dan tidak memiliki respon terhadap stimulis negatif yang dapat menghentikan pertumbuhan sel (Schultz & Harrington 2003).

Kebanyakan agen anti kanker seperti kemoterapi atau radiasi membunuh sel kanker melalui induksi kematian secara apoptosis (Fulda & Debatin 2003). Demikian pula senyawa aktif yang diperoleh dari tanaman seperti vinkristin dan vinblastin (Casado et al. 2007), taksol (Das et al. 2001), kurkumin (Tian et al.

2008), resveratrol (Pervaiz 2004) dan eurikomanon (Mahfudh et al. 2008) juga mempunyai aktivitas sitotoksik dan menginduksi kematian sel secara apoptosis.

Kegagalan pengaktifan apoptosis disebabkan adanya kecacatan pada program apoptosis atau berlebihnya isyarat pertumbuhan, dapat menyebabkan resistensi kanker (Fulda & Debatin 2003) (Fulda & Debatin 2006). Resistensi sel kanker adalah masalah utama dalam terapi kanser. Selain itu, pada kenyataannya agen-agen antikanker yang sedia juga menginduksi apoptosis pada sel normal ataupun sel kanser (tidak selektif). Oleh karena itu, objektif utama pengembangan antikanker adalah mendapatkan senyawa baru yang efektif dengan efek samping yang minimal.

Sesuatu agen kemoterapi atau agen antikanker baru perlu mempunyai enam ciri utama (Galati & O'Brien 2004) iaitu: tidak mempunyai efek toksik terhadap sel normal dan sel sehat, mempunyai efek toksik yang tinggi terhadap beberapa jenis kanker, aman untuk digunakan secara oral, mempunyai mekanisme efek samping yang diketahui, harga pasaran yang wajar dan diterima majoritas pengguna.

E. *Elephantopus scaber Linn* (Tapak Liman)

E. Elephantopus scaber dikenal di Indonesia sebagai Tapak Liman, di Sumatera: Tutup Bumi, di Jawa: Balangaduk, Jukut cancan, tapak liman, di Madura: tapak liman, tapak tana Wijayakusuma, 1995)

Taksonomi dari tanaman ini adalah:

Divisi	: Spermatophyta
Sub divisi	: Angiosperma
Kelas	: Dycotyledonae
Bangsa	: Asterales
Suku	: Asteraceae
Marga	: Elephantopus
Jenis	: *Elephantopus scaber,* L.

Tanaman tapak liman (*Elephantopus scaber* L) dilaporkan mengandung empat senyawa sesquiterpen lakton yaitu scabertopin (ES-2), isoscabertopin (ES-3), deoxyelephantopin (ES-4), isodeoxyelephantopin (ES-5) yang potensial sebagai antitumor (Xu et al. 2006). Kandungan lain yang berpotensi sebagai antikanker adalah deoxyelephantopin. elephantin, epifridelinol, stigmasterol, triacontan-1-ol, dotriacontan-1-ol, lupeol, lupeol acetat (Than et al. 2005). Isodeoksielephantopin juga dilaporkan memiliki efek menghambat siklus sel

(Kabeer et al. 2014). Tapak liman juga telah diketahui mempunyai efek terhadap sel-sel kanker payudara (Ho et al. 2011; Huang et al. 2010), sel kanker paru-paru (Farha et al. 2013), turunan sel kanker serviks (Listyowati & Nurkhasanah 2014) dan limfosit (Geetha et al. 2012).

Selain efek antikanker, tapak liman juga dilaporkan mempunyai efek antioksidan (Rout & Sahoo 2013). Efek antioksidan diharapkan dapat memperkuat efeknya sebagai antitumor.

F. Tanaman Kedelai

Kedelai atau Glycine max (L) Merr termasuk familia Leguminoceae, sub famili Papilionaceae, genus Glycine max, berasal dari jenis kedelai liar yang disebut Glycine unriensis (Samsudin, 1985). Menurut Ketaren (1986), secara fisik setiap kedelai berbeda dalam hal warna, ukuran dan komposisi kimianya. Perbedaan secara fisik dan kimia tersebut dipengaruhi oleh varietas dan kondisi dimana kedelai tersebut dibudidayakan. Biji kedelai tersusun atas tiga komponen utama, yaitu kulit biji, daging (kotiledon), dan hipokotil dengan perbandingan 8:90:2.

Glycine max merupakan tanaman asli daerah Asia subtropik seperti RRC dan Jepang Selatan, sementara Glycine soja merupakan tanaman asli Asia tropis di Asia Tenggara. Tanaman ini telah menyebar ke Jepang, Korea, Asia Tenggara dan Indonesia (Sulistiyani, 2009).

Klasifikasi Tanaman Kedelai :

Kindom : Plantae
Phylum : Tracheophyta
Class : Magnoliopsida
Orde : Fabales
Family : Fabaceae
Genus : Glycine willd
(Global Biodiversity Information Facility GBIF, 2010)

Komposisi kimia kedelai adalah 40,5% protein, 20,5% lemak, 22,2% karbohidrat, 4,3% serat kasar, 4,5% abu, dan 6,6% air. Kedelai merupakan sumber gizi yang sangat penting. Menurut Astuti (2003) dalam Anonim (2009b), komposisi gizi kedelai bervariasi tergantung varietas yang dikembangkan dan juga warna kulit maupun kotiledonnya. Kandungan protein dalam kedelai kuning bervariasi antara 31-48% sedangkan kandungan lemaknya bervariasi antara 11-21%. Antosianin kulit kedelai mampu menghambat oksidasi LDL kolesterol yang merupakan awal terbentuknya plak dalam pembuluh darah yang akan memicu

berkembangnya penyakit tekanan darah tinggi dan berkembangnya penyakit jantung koroner.

Kandungan lemak kedelai sebesar 18-20 % sebagian besar terdiri atas asam lemak (88,10%). Selain itu, terdapat senyawa fosfolipida (9,8%) dan glikolipida (1,6%) yang merupakan komponen utama membran sel. Kedelai merupakan sumber asam lemak essensial linoleat dan oleat (Smith and Circle, 1978). Protein kedelai mengandung 18 asam amino, yaitu 9 jenis asam amino esensial dan 9 jenis asam amino nonesensial. Asam amino esensial meliputi sistin, isoleusin, leusin, lisin, metionin, fenil alanin, treonin, triptofan dan valin. Asam amino nonesensial meliputi alanin, glisin, arginin, histidin, prolin, tirosin, asam aspartat dan asam glutamat.

Hasil penelitian terdahulu, Ekstrak Etanol Biji Kedelai Detam 1 terbukti mengandung fenolik, flavonoid H2 SO4 triterpenoid, steroid, saponin, kuinon dan tanin, namun tidak mengandung alkaloid.

G. Tanaman Temulawak

Saat ini, sebagian besar budidaya temulawak berada di Indonesia, Malaysia, Thailand, dan Filipina. Tanaman ini selain di Asia Tenggara dapat ditemui pula di China, Indochina, Bardabos, India, Jepang, Korea, Amerika Serikat dan Beberapa negara Eropa. Nama daerah di Jawa yaitu temulawak, di Sunda disebut koneng gede, sedangkan di Madura disebut temulabak. Tanaman ini dapat tumbuh dengan baik pada dataran rendah sampai ketinggian 1500 meter di atas permukaan laut dan berhabitat di hutan tropis. Rimpang temu lawak dapat tumbuh dan berkembang dengan baik pada tanah yang gembur (Kartika, 2010).

Klasifikasi Tanaman Temulawak :

Kingdom : Plantae
Divisi : Spermathophyta
Subdivisi : Angiospermae
Kelas : Monocotyledoneae
Bangsa : Scitamineae
Famili : Zingiberaceae
Marga : Curcuma
Spesies : Curcuma xanthorhiza roxb
(Afifah, 2003)

Temulawak atau dalam Bahasa Inggris disebut java turmeric ini, secara tradisional digunakan untuk menyembuhkan penyakit perut, hati, konstipasi, pembuluh darah pecah, demam anak-anak, kulit kasar, disentri dan sebagainya.

Dilaporkan **curcuma xanthorrhizol** juga memiliki kemampuan antitumor, **anti kanker**, **anti diabetes**, hipotriceriakademik, anti inflamantori, **hepatoprotective**, anti mikroba, dan anti lemak (Salim, 2009).

BAB III

TUJUAN DAN MANFAAT PENELITIAN

A. Tujuan penelitian

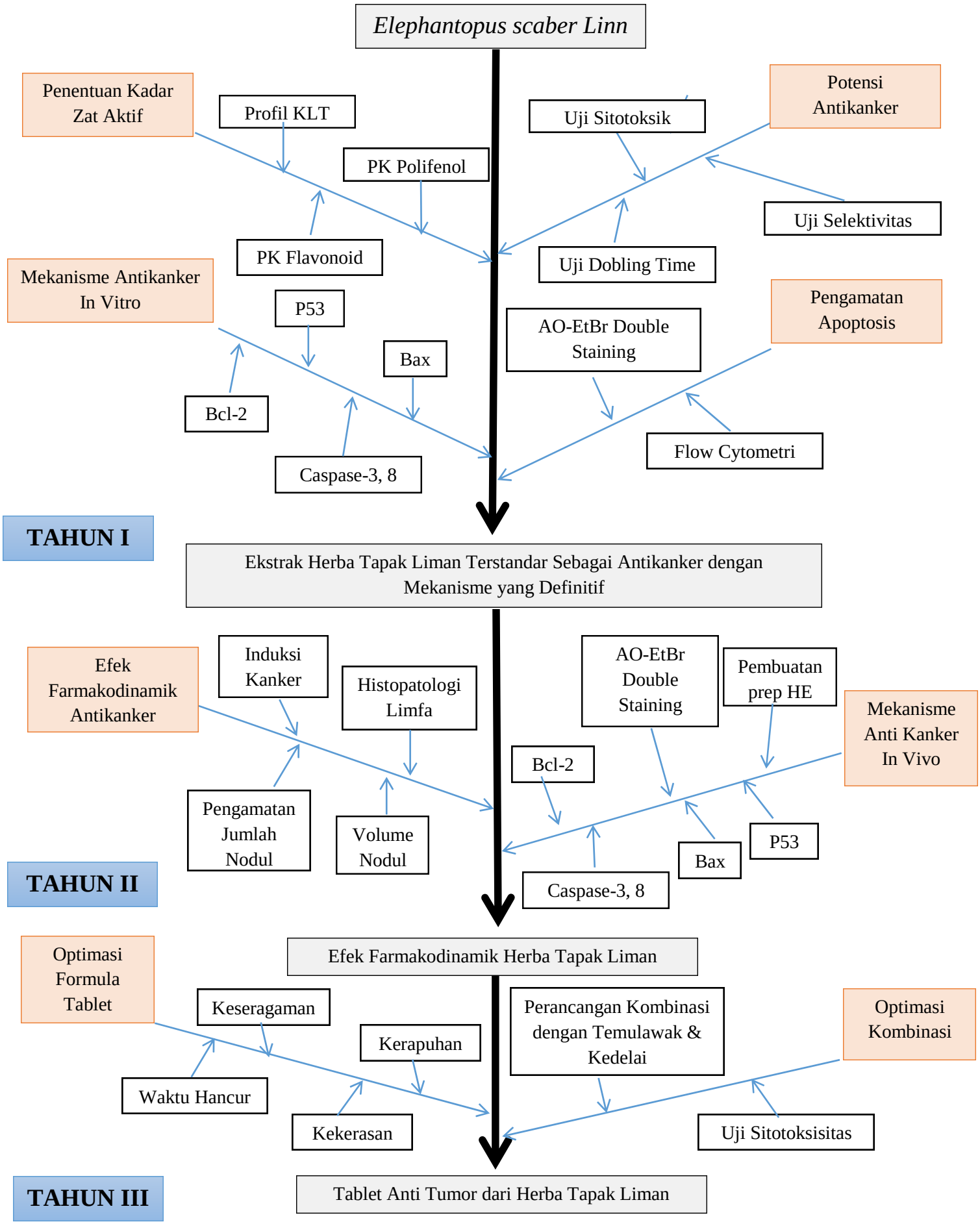
1. Mengkaji potensi herba tapak liman sebagai antikanker payudara dengan mengamati potensi ketoksikan (IC50) dan mekanisme pemacuan apoptosis.
2. Mengkaji mekanisme antikanker dengan melihat ekspresi protein-protein p53, Bax, Bcl-2, caspase-6 dan caspase-7 secara in vitro terhadap sel kanker payudara T47D.
3. Mengkaji efek kemoprefentif herba tapak liman pada hewan uji yang diinduksi DMBA terhadap timbulnya nodul tumor, ekspresi protein p53, Bax, Bcl-2, caspase-6 dan caspase-7 pada jaringan payudara.
4. Mengkaji potensi herbal Tapak Liman dengan beberapa tanaman obat lain sebagai Anti kanker payudara dengan mengamati potensi ketoksikan (IC50)
5. Mengembangkan formulasi produk antitumor herba tapak liman melalui kombinasi dengan herba lain untuk mendapatkan efek sinergi.
6. Mengembangkan produk antitumor sebagai sediaan tablet untuk mendapatkan sediaan yang praktis digunakan.

B. Manfaat penelitian

1. Hasil penelitian akan menjadi dasar bagi pengembangan herba tapak liman menjadi agen antikanker yang mempunyai bukti ilmiah (evidence based herbal medicines).
2. Hasil penelitian juga akan memberi nilai tambah secara ekonomi terhadap herba tapak liman sebagai antitumor.

BAB IV
METODE PENELITIAN

Penelitian yang akan diusulkan pada penelitian ini bertujuan untuk mendapatkan perbandingan kombinasi herbal Tapak Liman dengan tanaman obat lain yang menghasilkan efek kemoprefentif yang optimal serta mendapatkan formula tablet antitumor dari ekstrak herbal tapak liman dengan bahan-bahan alami yang dapat berfungsi memberikan efek sinergis sebagai antitumor. Secara umum, penelitian yang akan dilakukan pada tiga tahun dapat dilihat pada gambar 1.


Elephantopus scaber Linn
Penentuan Kadar Zat Aktif
Profil KLT
PK Polifenol
PK Flavonoid
Potensi Antikanker
Uji Sitotoksik
Uji Selektivitas
Uji Dobling Time
Mekanisme Antikanker In Vitro
P53
Bax
Bcl-2
Caspase-3, 8
AO-EtBr Double Staining
Pengamatan Apoptosis
Flow Cytometri
TAHUN I
Ekstrak Herba Tapak Liman Terstandar Sebagai Antikanker dengan Mekanisme yang Definitif
Efek Farmakodinamik Antikanker
Induksi Kanker
Histopatologi Limfa
Pengamatan Jumlah Nodul
Volume Nodul
AO-EtBr Double Staining
Pembuatan prep HE
Mekanisme Anti Kanker In Vivo
Bcl-2
Bax
P53
Caspase-3, 8
TAHUN II
Efek Farmakodinamik Herba Tapak Liman
Optimasi Formula Tablet
Keseragaman
Kerapuhan
Waktu Hancur
Kekerasan
Perancangan Kombinasi dengan Temulawak & Kedelai
Optimasi Kombinasi
Uji Sitotoksisitas
TAHUN III
Tablet Anti Tumor dari Herba Tapak Liman

Gambar 1 Penelitian Pengembangan Tapak Liman sebagai Antikanker Payudara

A. Tahun I

Tujuan **:** Mengkaji mekanisme tapak liman sebagai antikanker payudara secara in vitro

Bahan Utama : Ekstrak herba tapak liman (*Elephantopus scaber Linn*)

Objek Uji : Turunan sel kanker payudara (T47D).

Desain Penelitian : Penelitian ini merupakan penelitian eksperimental laboratorium untuk menguji mekanisme ekstrak tapak liman pada turunan sel kanker payudara.

Jalannya Penelitian

Ekstraksi herba tapak liman

Herba tapak liman diekstraksi menggunakan etanol, dan diuapakan menggunakan rotary evaporator sehingga diperoleh ekstrak kental.

Penetapan kadar flavonoid dan polifenol

Penetapan kadar flavonoid total dilakukan dengan menggunakan standar quersetin dengan pembentukan komplek dengan ALCl3 dan ditetapkan kadarnya menggunakan spektrofotometri (Chang et al. 2002) dan kandungan polifenol total ditetapkan dengan reagen Folin Ciocalteu berdasarkan reaksi reduksi dan selanjutnya ditentukan kadarnya dengen spektrofotometri menggunakan asam galat sebagai standar.

Uji Sitotoksisitas

Uji sitotoksisitas dilakukan dengan metode MTT berdasarkan reduksi MTT oleh enzim NADH dari sel hidup. Persentase sel yang hidup dibandingkan terhadap kontrol. Parameter sitotoksisitas eurikomanon dinyatakan sebagaii IC50

Uji sitotoksik dilakukan dengan cara menginkubasi 100 µl suspensi sel Hela dengan kepadatan 1x104/100 µl dalam inkubator CO2 5% dan 100 µl seri kadar ekstrak etanol daun tapak liman 4000 2000, 1000, 500, 250, 125, 62,5, dan 31,125 µg/ml. Kontrol sel berisi 100 µl suspensi sel dan 100 µl media komplit. Kontrol media berisi 200 µl media komplit. Kontrol pelarut berisi 100 µl DMSO dalam berbagai kadar sesuai yang diujikan dan 100 µl media komplit.

Sel didistribusikan ke dalam 96 sumuran dan diinkubasi bersama ekstrak uji selama 24 jam. Pada masing-masing sumuran ditambah 100µl MTT 5mg/ml dalam PBS. Selanjutnya diinkubasi lagi 4 jam pada suhu 37o C. Sel yang hidup akan bereaksi dengan MTT membentuk warna ungu. Reaksi MTT dihentikan dengan SDS 10% (*reagen stopper*),

lalu diinkubasi semalam pada suhu kamar. Serapan dibaca pada ELISA *reader* pada panjang gelombang 550 nm.

Pengamatan Apoptosis

Pengamatan apoptosis pada penelitian ini dilakukan menggunakan pewarnaan akridin orange-propidium iodida. Hasil perhitungan IC50 digunakan untuk menentukan kadar apoptosis. Kadar yang dibuat untuk apoptosis yaitu dua kali nilai IC50, nilai IC50 dan setengah dari IC50. Sel Hela ditanam pada *coverslips* yang dimasukan dalam microplate 24 sumuran sehingga diperoleh kepadatan 5 X 104 sel/sumuran dan diinkubasi sampai 50-60% konfluen. Setelah itu diinkubasi dengan senyawa uji selama 24 jam. Medium diambil, dicuci dengan PBS. *Cover slip* yang memuat sel diangkat, diletakan di atas *object glass* dan ditambahkan 10 μL 1x *Working Solution* etidium bromida-akridin orange kemudian sel segera diamati di bawah mikroskop flouresens (Zeiss MC 80). Sel hidup berfluoresensi hijau (dengan akridin oranye) dan sel mati berfluoresensi merah (dengan etidium bromida).

Uji Pengamatan Waktu Penggandaan Sel

Uji dilakukan dengan menghitung jumlah sel setelah diberi perlakuan setiap satuan waktu (setiap 24 jam). Jumlah sel dihitung secara langsung dengan haemositometer kemudian dibuat kurva jumlah sel vs waktu inkubasi. Perbedaan waktu penggandaan sel dilihat dari slope pada kurva atau dihitung dengan ekstrapolasi.

Uji Mekanisme Apoptosis

Uji mekanisme dilakukan dengan mengamati ekspresi protein p53, Bax, Bcl-2, kaspase-3, kaspase-8 dan protein sasaran apoptosis DFF45. Pengamatan dilakukan menggunakan teknik imunositokimia. Sel (kepadatan 1,5 X 104 sel/sumuran) ditanam pada *plate* 24 sampai 70 % konfluen. Sehari sebelum perlakuan medium diganti dengan medium RPMI 1640. Setelah itu diinkubasi dengan ekstrak uji selama 24 jam. Sel yang telah diinkubasi dipanen dan dibuat apusan pada gelas obyek (*poly-l-lysine slide*). Preparat difiksasi dengan aseton. Preparat diletakkan dalam *normal mouse serum* (1:50) selama 15 menit. Dibuang (tanpa cuci), lalu ditetesi dengan Primer Antibodi Monoklonal anti p53, Bax, kaspase-3, kaspase-8 dan DFF45. Preparat diinkubasi dalam biotin selama 10 menit dan dicuci dengan PBS sebanyak 2 kali selama 5 menit. Kemudian preparat diinkubasi dalam *streptavidin-peroksidase* selama 10 menit dan dicuci dengan PBS sebanyak 2 kali selama 5 menit. Preparat diamati dengan mikroskop dan intensitas warna dibandingkan dengan kontrol.

B. Tahun II

Tujuan	**:** Mengkaji efek kemopreventif ekstrak tapak liman pada karsinogenesis kanker payudara menggunakan induksi DMBA
Bahan Utama	: Ekstrak herba tapak liman (*Elephantopus scaber Linn*)

Objek Uji : Tikus betina galur Sprague Dawley

Desain Penelitian : Penelitian ini merupakan penelitian eksperimental laboratorium untuk menguji efek kemoprefentif ekstrak herba tapak liman terhadap karsinogenesis oleh DMBA

Jalannya Penelitian

Induksi hewan uji menggunakan DMBA

Induksi kanker payudara dilakukan menggunakan DMBA menggunakan metode yang telah (Flesher & Sydnor 1971). Hewan uji diberi perlakuan DMBA doses 1mg dalam minyak wijen. Pemberian dilakukan 20 kali dengan injeksi subcutan pada punggung. Evaluasi terbentuknya kanker payudara dilakukan setelah 30 hari pemberian.

Perlakuan hewan uji

Setelah induksi DMBA hewan uji diberi perlakuan ekstrak herba tapak liman. Dengan pengelompokan Grup I: baseline, Grup II: control negative, grup III, IV dan V adalah grup perlakuan ekstrak herba tapak liman dengan dosis 50, 100 dan 200mg/kg BB sekali sehari.

Analisis terbentuknya tumor

Penilaian analisis terbentuknya tumor dilihat dari jumlah nodul dan volume nodul yang terbentuk pada tiap kelompok.

Kajian mekanisme farmakodinamik molekuler kemoprefensi ekstrak herba tapak liman

Kajian mekanisme dilakukan terhadap preparat irisan histology organ payudara. Pengamatan meliputi pengamatan level apoptosis dengan pengecatan akridin orange-etidium bromide dan mekanisme apoptosis yaitu ekspresi protein p53, Bax, Bcl-2 dan caspase-3 yang diamati dengan teknik imunohistokimia menggunakan antibody yang spesifik untuk masing-masing protein.

C. Tahun III

Tujuan **:** Formulasi sediaan kemoprevensi antitumor dari herba tapak liman dan uji sitotoksik ekstrak herbal tapak liman dengan berbagai tanaman obat lain terhadap kanker payudara (T47D).

Bahan Utama : Ekstrak herba tapak liman (*Elephantopus scaber Linn*), ekstrak herbal kedelai (*Glycine max*), ekstrak herbal temulawak (*Curcuma xanthorriza)*

Desain Penelitian : Penelitian ini merupakan penelitian eksperimental Laboratorium untuk mendapatkan formula antikanker dari herba tapak liman sebagai bahan utama, dan untuk mendapatkan perbandingan kosentrasi herbal tapak liman dengan berbagai tanaman obat lain sebagai kemopreventif kanker payudara yang optimal.

Jalannya Penelitian

Optimasi formula antitumor

Optimasi formula kombinasi herba antitumor dimaksudkan untuk mendapatkan formula yang lebih baik dengan efek sinergis dari kombinasi beberapa herba yang sudah diketahui mempunyai efek antitumor. Kombinasi akan dilakukan antara herba tapak liman, temu lawak dan kedelai. Temu lawak telah dilaporkan mempunyai efek antikanker pada kanker paru (Choi et al. 2005), kulit (Chung et al. 2007) dan hati (Hong et al. 2005). Kandungan isoflavon dari kedelai juga telah dilaporkan mempunyai efek kemoprevensi terhadap kanker (Sarkar & Li 2002; Sarkar & Li 2003). Dalam formulasi, kedelai selain diharapkan meningkatkan efek antitumor melalui efek sinergi juga diharapkan berfungsi sebagai bahan pengisi.

Formula bahan aktif akan dibuat dengan mengubah komposis ekstrak herba tapak liman dan temu lawak. Formula yang optimum akan dinilai berdasarkan toksisitasnya terhadap sel T47D.

Optimasi formula tablet

Formulasi tablet akan dilakukan dengan metode granulasi basah. Formula yang diperoleh akan dievaluasi dengan penilaian sifat-sifat fisik tablet meliputi uji kekerasan, kerapuhan, waktu hancur dan keseragaman bobot tablet.

Uji Sitotoksik

Uji sitotoksisitas dilakukan dengan metode MTT berdasarkan reduksi MTT oleh enzim NADH dari sel hidup. Persentase sel yang hidup dibandingkan terhadap kontrol. Parameter sitotoksisitas dinyatakan sebagaii $IC_{50.}$

Uji sitotoksik dilakukan dengan cara menginkubasi 100 µl suspensi sel T47D dengan kepadatan $1x10^4$/100 µl dalam inkubator CO_2 5% dan 100 µl seri kadar ekstrak etanol daun tapak liman kombinasi kedelai, dan temulawak 500, 250, 125, 62,5, dan 31,125 µg/ml. Kontrol sel berisi 100 µl suspensi sel dan 100 µl media komplit. Kontrol media berisi 200 µl media komplit. Kontrol pelarut berisi 100 µl DMSO dalam berbagai kadar sesuai yang diujikan dan 100 µl media komplit.

Sel didistribusikan ke dalam 96 sumuran dan diinkubasi bersama ekstrak uji selama 24 jam. Pada masing-masing sumuran ditambah 100µl MTT 5mg/ml dalam PBS.

Selanjutnya diinkubasi lagi 4 jam pada suhu 37^{o} C. Sel yang hidup akan bereaksi dengan MTT membentuk warna ungu. Reaksi MTT dihentikan dengan SDS 10% (*reagen stopper*), lalu diinkubasi semalam pada suhu kamar. Serapan dibaca pada ELISA *reader* pada panjang gelombang 550 nm.

BAB V
HASIL DAN LUARAN YANG DICAPAI

A. Preparasi Sampel

Daun Tapak Liman (*Elephantopus scaber* Linn) yang digunakan sebagai bahan baku utama dalam penelitian ini dibersihkan dengan menggunakan air mengalir dan dikeringkan dengan menggunakan bantuan oven selama kurang lebih 1 hari untuk menurunkan kadar air dari daun tapak liman. Selanjutnya dilakukan perhitungan kandungan air (%MC) setelah dilakukan pengeringan didalam oven. Diperoleh kandungan air dalam sampel daun Tapak Liman (*Elephantopus scaber* Linn) adalah kurang dari 10%, dimana kadar tersebut memenuhi syarat keberterimaan kandungan air dari suatu sampel daun tapak liman yaitu kurang dari 10%.

Kemudian sampel daun tapak liman diserbuk secara manual. Setelah itu diayak dengan menggunakan ayakan mesh 20/40 dan serbuk yang lolos pada ayakan no 20 serta tidak lolos ayakan no 40 yang digunakan untuk ektraksi.

Selanjutnya preparasi rimpang temulawak (curcuma xanthorizza) yang digunakan dalam penelitian ini, di bersihkan dari kulitnya lalu di cuci dengan air mengalir dan di rajang kecil-kecil, kemudian di keringkan dengan menggunkan oven selama kurang lebih 1 hari untuk menurunkan kadar air dari rimpang temulawak.

Kemudian sampel rimpang temulawak di serbuk dengan menggunkan blender. Setelah itu diayak dengan menggunkan ayakan mesh 20/40 dan serbuk yang lolos pada ayakan no 20 serta tidak lolos ayakan no 40 yang digunakan untuk ektraksi. Sedangkan biji kedelai (glycine max) yang juga di gunakan sebagai bahan dalam penelitian ini, di serbuk dengan menggunakan blender. Setelah itu diayak dengan menggunkan ayakan mesh 20/40 dan serbuk yang lolos pada ayakan no 20 serta tidak lolos ayakan no 40 yang digunakan untuk ektraksi.

B. Ekstraksi daun tapak liman

Simplisia serbuk daun tapak liman sebanyak 500 gram diekstraksi menggunakan pelarut etanol 70% sebanyak 5000 ml dengan metode maserasi berulang dengan pengocokan sesekali atau menggunakan bantuan pengaduk mekanik. Tahapan ini dilakukan selama 2-3 hari lalu disaring sehingga diperoleh ekstrak cair. Ekstrak cair lalu dievaporasi menggunakan *vacuum rotary evaporator* sehingga diperoleh ekstrak kental daun tapak liman.

C. Ekstraksi rimpang temulawak

Simplisia rimpang temulawak sebanyak 500 gram, diekstraksi menggunakan pelarut etanol 70% sebanyak 5000 ml dengan metode maserasi berulang dengan pengocokan sesekali atau menggunakan bantuan pengaduk mekanik. Tahapan ini dilakukan selama 2-3 hari lalu disaring sehingga diperoleh ekstrak cair. Ekstrak cair lalu dievaporasi menggunakan *vacuum rotary evaporator* sehingga diperoleh ekstrak kental rimpang temulawak.

D. Ekstraksi kedelai

Simplisia biji kedelai sebanyak 500 gram diekstraksi menggunakan pelarut etanol 70% sebanyak 5000 ml dengan metode maserasi berulang dengan pengocokan sesekali atau menggunakan bantuan pengaduk mekanik. Tahapan ini dilakukan selama 2-3 hari lalu disaring sehingga diperoleh ekstrak cair. Ekstrak cair lalu dievaporasi menggunakan *vacuum rotary evaporator* sehingga diperoleh ekstrak kental biji temulawak.

E. Uji Sitotoksisitas

Pada penelitian ini menggunakan metode MTT karena telah terbukti lebih sensitif, cepat dan akurat dibandingkan dengan metode perhitungan langsung (Doyle and Griffit,2000). Pada metode MTT juga memiliki kekurangan, yaitu sampel yang berwarna dapat memberikan absorbansi, sehingga absorbansi yang terbaca tidak hanya warna ungu, yang sebanding dengan jumlah sel hidup tetapi juga warna dari sampel. Hal ini dapat diatasi dengan cara menggunakan kontrol sampel yang berwarna sehingga hasil lebih valid.

Penetapan dengan metode MTT ini merupakan pengukuran Kolorimetri yang didasarkan pada terjadinya pembentukan garam Formazan yang merupakan zat warna ungu dan tidak larut air. Reagen MTT akan bereaksi dengan sel hidup dan akan pecah menjadi garam Formazan oleh enzim reduktase suksinat tetazolium yang termasuk dalam rantai respirasi Mitokondria dan hanya aktif pada sel hidup.

Kemudian suspensikan larutan seri kadar yang dibuat untuk masing-masing sampel replikasi 3 kali sebanyak 100 µl kedalam sumuran termasuk pada kontrol sel, kecuali pada sumuran kontrol media. Terlebih dahulu plate ditiriskan atau dibuang larutan didalamnya dengan meniriskan diatas tisu lembut. Setelah itu di inkubasi kembali selama 24 jam kedalam incubator CO_2.

Uji sitotoksik dilakukan dengan cara menginkubasi 100 µl suspensi sel T47D kedalam 84 sumuran dan Kontrol sel berisi 100 µl suspensi sel dalam media komplit sebanyak 6 sumuran. Sedangkan kontrol media sebanyak 6 sumuran sisa sementara dikosongkan terlebih dahulu, kemudian diinkubasi selama 24 jam didalam inkubator CO_2.

Selanjutnya pembuatan larutan uji, yaitu sebanyak 10 mg sampel masing-masing perlakuan (Perbandingan Ekstrak etanol daun tapak liman, rimpang temulawak dan kedelai) dilarutkan kedalam 100 µl DMSO kedalam clonical tube, dari sampel tersebut dibuat seri larutan kadar dimulai dari 1500; 750; 375; 187,5 ; 93,75 dan 46,875 µg/ml.

Tabe1 1. Perbandingan Bahan Yang Digunakan Dalam Pembuatan Sampel

Kode	Perbandingan		
	Kedelai	Tapak Liman	Temulawak
A	1	1	
B	1	2	
C	2	1	
D	1	3	
E	3	1	
F		1	1
G		1	2
H		2	1
I		1	3
J		3	1
K	1	1	1
L	1	1	2
M	1	2	1
N	2	1	1

Reaksi antara reagen MTT dengan enzim reduktase suksinat tetazolium merupakan proses enzimatis yang berlangsung kontinue. Sehingga diperlukan reagen stopper (SDS 10% dalam HCl 0,01 N) bertujuan untuk menghentikan reaksi ezimatis, kemudian diinkubasi selama 24 jam tujuannya agar kristal MTT larut. Intensitas warna ungu ditetapkan secara Spektrofotometri dengan

ELISA reader pada panjang gelombang 550nm. Absorbansi yang diperoleh digunakan untuk menghitung % sel hidup T47D .

Hasil inkubasi selama 24 jam di amati dibawah mikroskop untuk melihat pertumbuhan sel. Pada masing-masing sumuran ditambah 100µl MTT 5mg/ml dalam PBS. Selanjutnya diinkubasi lagi 4 jam pada suhu 37^{o} C. pengamatan diibawah mikroskop untuk memastikan sel yang hidup, Sel yang hidup akan bereaksi dengan MTT membentuk warna ungu. Kemudian reaksi MTT dihentikan dengan penambahan SDS 10% (*reagen stopper*) , lalu diinkubasi semalam pada suhu kamar dengan dibungkus kertas gelap atau alumunium foil. Keesokan harinya serapan dibaca pada ELISA *reader* pada panjang gelombang 550 nm dan didapat data absorbansi pada masih masing sampel, kontrol sel dan kontrol media untuk digunakan dalam analisis perhitungan IC50.

Dari hasil data absorbansi yang didapat ELISA reader , pada absorbansi sampel kombinasi ekstrak Ethanol daun tapak liman dengan temulawak dan kedelai didapatkan bahwa tidak terdapat perbedaan yang begitu bermakna pada konsentrasi 1500 hingga 46,875 ppm.

Dari penelitian sebelumnya diperoleh nilai IC 50 sebesar 132,173 ppm. Sedangkan Pada penelitian terhadap Ekstrak ethanol daun Tapak liman ini diperoleh IC 50 sebsar 238,506 ppm, sehingga dapat dikatakan ekstrak ethanol Daun Tapak Liman ini memiliki aktifitas sitotoksik yang kurang baik trhadap sel T47D, karena menurut Meiyanto et al, (2008) ekstrak uji yang memiliki kadar IC50 di bawah 100 ppm menunjukkan adanya potensi sebagai agen kemopreventif. Namun, bukan berarti Ekstrak tumbuhan yang memiliki IC50 >100 ppm tidak berpotensi untuk dikembangkan sebagai Antikanker, karena menurut Machana, 2011 ekstrak dikatakan tidak aktif sebagai Antikanker jika memiiki nilai IC50 > 500 ppm.

Dari hasil data absorbansi yang didapat ELISA reader , pada absorbansi sampel kombinasi ekstrak Ethanol daun tapak liman dengan temulawak dan kedelai didapatkan bahwa tidak terdapat perbedaan yang begitu bermakna pada konsentrasi 1500 hingga 46,875 ppm.

Selanjutnya dilakukan perhitungan kadar IC50 dari keseluruhan sampel perlakuan, dengan cara membuat kurva hubungan antara log konsentrasi vs % sel hidup kemudian didapat data IC50 masing-masing sampel perlakuan dan dibuat kedalam bentuk (antilog) IC50 untuk mendapatkan kadar IC50 yang diinginkan dalam satuan ppm. Data konsentrasi kadar IC50 pada perlakuan kombinasi ektrak etanol daun tapak liman dan temulawak adalah sebesar 171,93ppm pada perbandingan 3:1, sedangkan data konsentrasi kadar IC50 pada masing-masing sampel perlakuan dari kombinasi ektrak etanol daun tapak liman dan kedelai adalah sebesar 159,423ppm pada perbandingan 1:3. Data konsentrasi kadar IC50 pada masing-masing sampel perlakuan dari kombinasi ektrak etanol daun tapak liman, kedelai, dan temulawak adalah 163,88ppm pada perbandingan 1:2:1.

Dari perbandingan ekstrak Tunggal dan ekstrak Kombinasi, terlihat bahwa nilai IC 50 yang diperoleh tidak jauh berbeda, dimana nilai IC50 ektrak kombinasi lebih kecil daripada nilai IC50 ekstrak tungggal, meskipun perbedannya tidak terlalu signifikan, hal ini dapat menjelaskan bahwa kombinasi ektrak Daun Tapak liman dan ekstrak Kedelai maupun ekstrak Temulawak tidak terlalu berpengaruh terhadap proses kematian sel T47D yang dapat dibuktikan dengan nilai IC50 yang perbedaannya tidak terlalu signifikan.

Berikut data kadar Absorbansi dan hasil perhitungan IC50 yang diperoleh dari keseluruhan masing-masing sampel perlakuan.

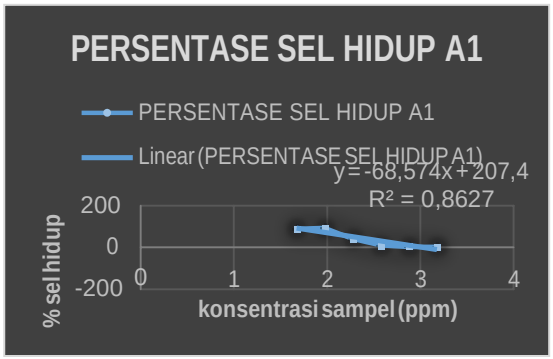
Tabel 2 kadar Absorbansi dan hasil perhitungan IC50 Ekstrak Kombinasi

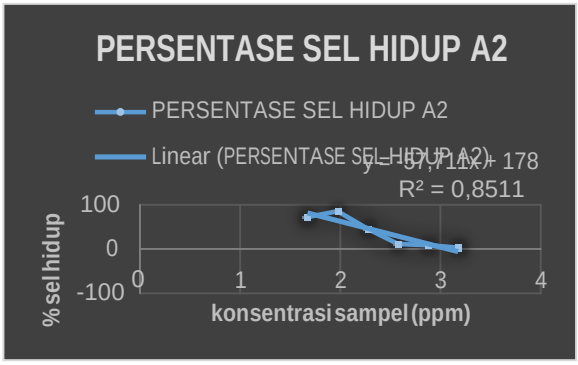
Konsentrasi sampel	Absorbansi											
	A			B			C			D		
1500 ppm	0.1212	0.1211	0.1221	0.1275	0.1243	0.1384	0.158	0.1601	0.162	0.1155	0.1254	0.1309
750 ppm	0.1415	0.1518	0.1618	0.1621	0.1666	0.1716	0.2125	0.2014	0.2085	0.1642	0.1458	0.1534
375 ppm	0.1606	0.1689	0.1877	0.1772	0.1797	0.1948	0.3166	0.4427	0.385	0.2439	0.2156	0.2221
187,5 ppm	0.3384	0.3609	0.4252	0.5146	0.5356	0.5036	0.5154	0.6104	0.5562	0.2979	0.3368	0.2843
93,75 ppm	0.6317	0.5842	0.6165	0.5931	0.6342	0.6734	0.6493	0.6185	0.5604	0.6021	0.5754	0.6087
46,875 ppm	0.6092	0.5114	0.62	0.6489	0.6809	0.6463	0.6747	0.6544	0.7021	0.6228	0.6029	0.6428

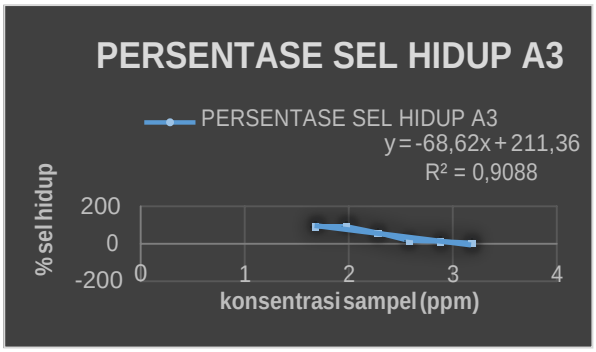
Kontrol sel	0,7428	0,6444	0,6122	Rata-rata kontrol sel	0,666467
Kontrol media	0,0959	0,1022	0,1037	Rata-rata kontrol media	0,1006

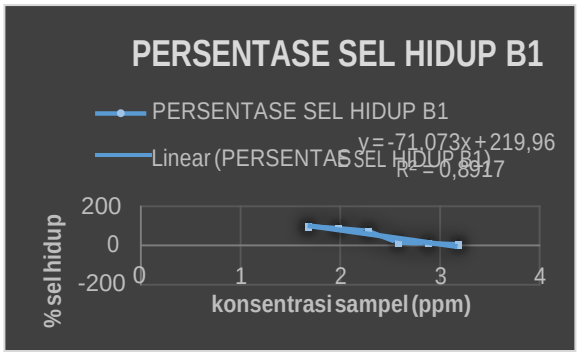
persentase sel hidup (%) A1	persentase sel hidup (%) A2	persentase sel hidup (%) A3	persentase sel hidup (%) B1	persentase sel hidup (%) B2	persentase sel hidup (%) B3
3.640434	3.622762	3.799482	4.75377	4.188266	6.680019
7.227851	9.048068	10.81527	10.86828	11.66352	12.54713
10.6032	12.06998	15.39232	13.53676	13.97856	16.64703
42.02403	46.00024	57.36334	73.16211	76.87323	71.21819
93.85603	85.46183	91.16989	87.03464	94.29783	101.2253
89.87983	72.59661	91.78841	96.89562	102.5507	96.43615
persentase	persentase	persentase	persentase	persentase	persentase

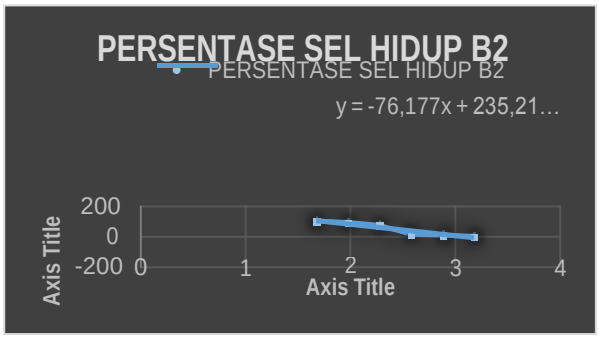
sel hidup (%) C1	sel hidup (%) C2	sel hidup (%) C3	sel hidup (%) D1	sel hidup (%) D2	sel hidup (%) D3
10.14373	10.51484	10.85061	2.633129	4.382658	5.354618
19.77498	17.81338	19.0681	11.2394	7.987747	9.33082
38.17154	60.45594	50.25919	25.32399	20.32281	21.47149
73.30349	90.09189	80.51367	90.09189	41.74128	32.46348
96.96631	91.52333	81.25589	88.62512	83.90669	89.79147
101.455	97.86758	106.2971	92.28322	88.76649	95.81762

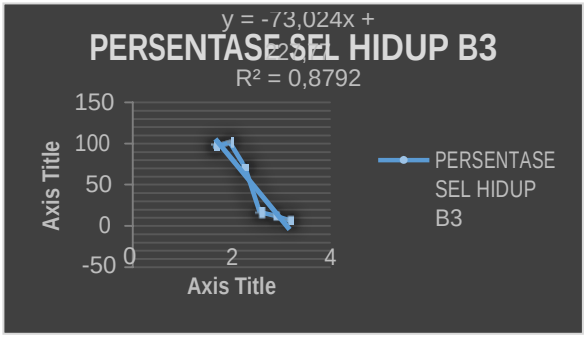


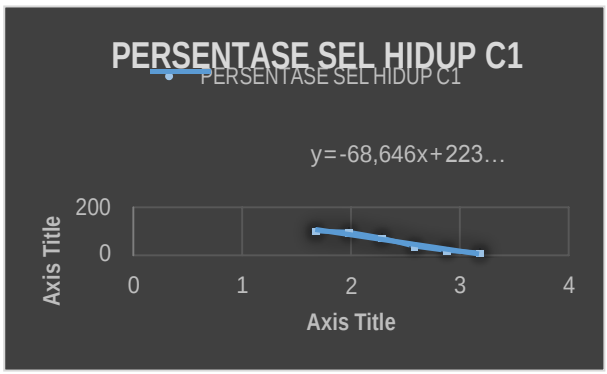


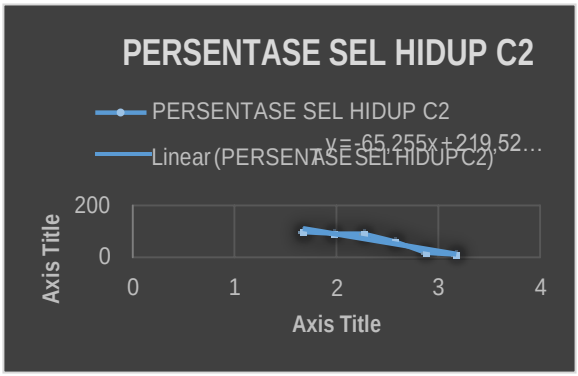


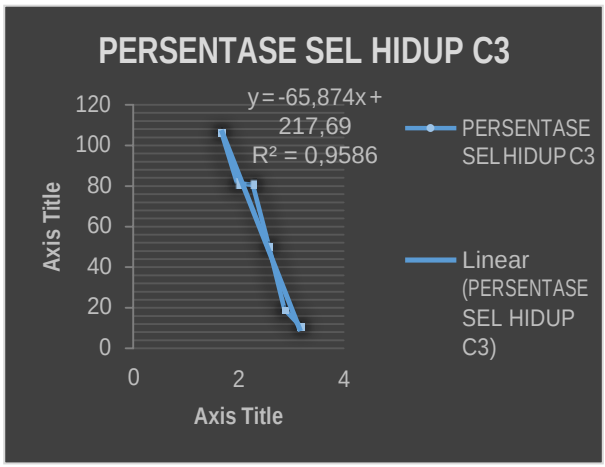


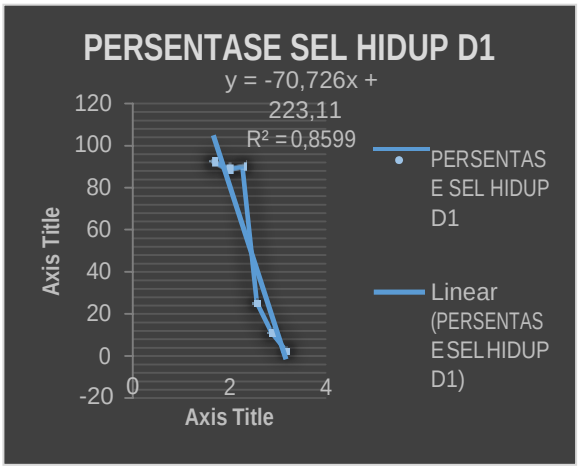


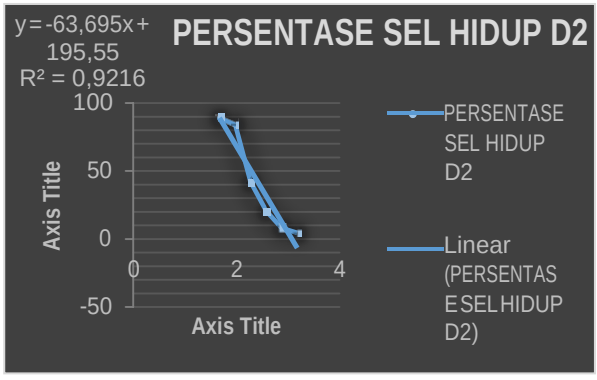


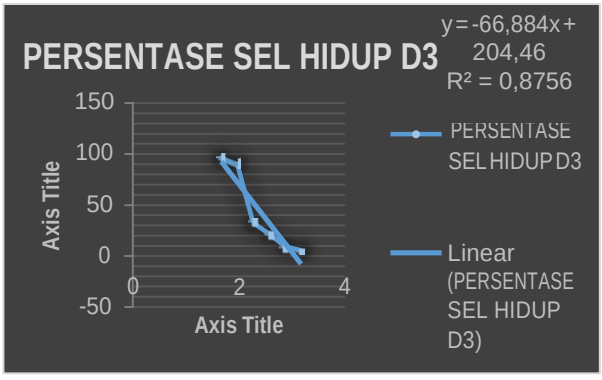






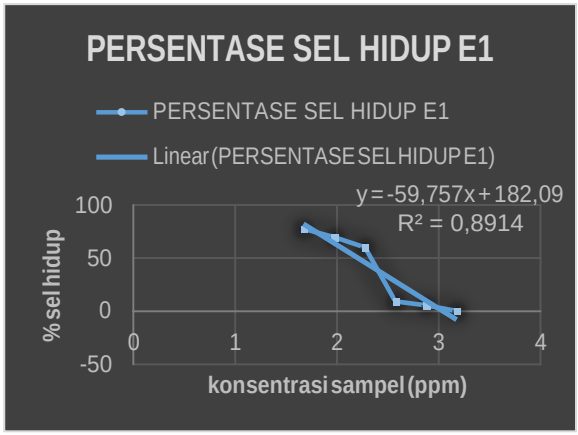


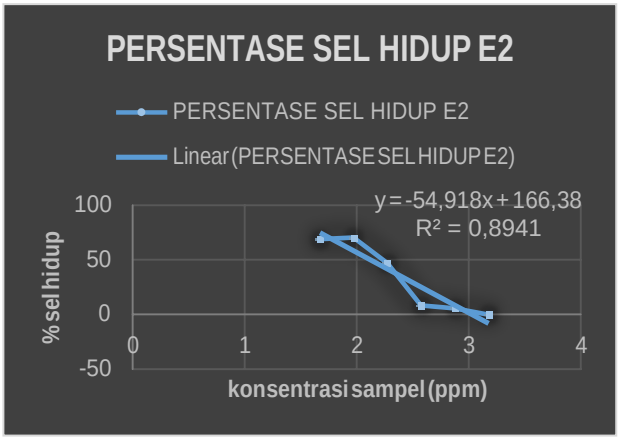


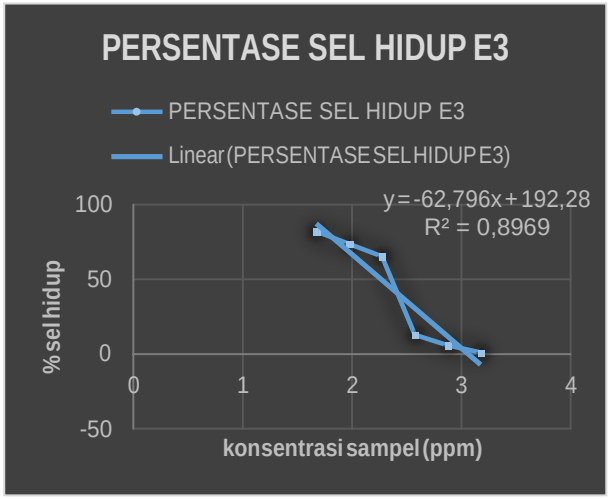


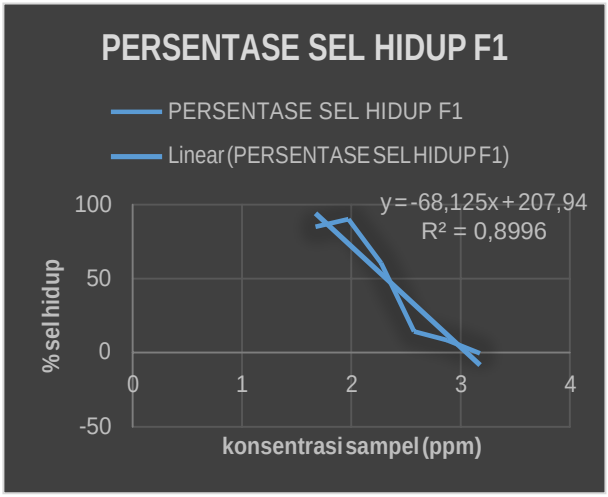
Absorbansi											
E			F			G			H		
0.1027	0.0996	0.1061	0.0974	0.1043	0.107	0.178	0.1804	0.192	0.1371	0.1412	0.142
0.1337	0.1324	0.133	0.1481	0.1511	0.149	0.1974	0.1893	0.1919	0.1584	0.1641	0.164
0.1542	0.1464	0.1736	0.1805	0.1891	0.179	0.3998	0.439	0.5061	0.2103	0.2142	0.202
0.4422	0.3661	0.4699	0.4422	0.4446	0.460	0.4547	0.4955	0.5134	0.3082	0.5043	0.500
0.4961	0.4984	0.5172	0.6094	0.5632	0.57	0.5784	0.5593	0.5433	0.5404	0.54	0.509
0.5402	0.4909	0.5651	0.5806	0.6315	0.67	0.6288	0.7227	0.628	0.5426	0.5318	0.460

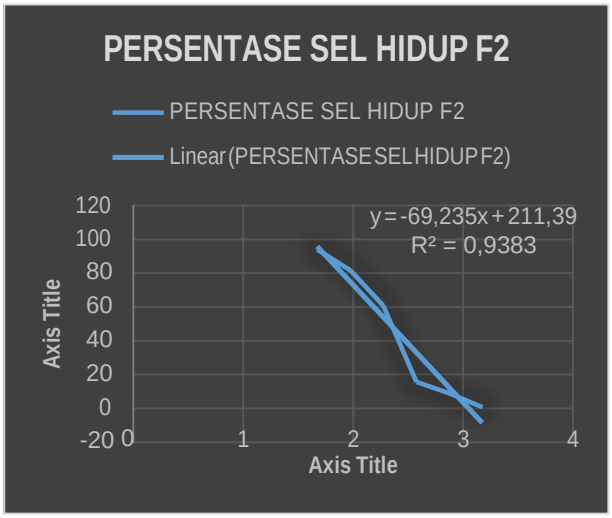
persentase sel hidup (%) E1	persentase sel hidup (%) E2	persentase sel hidup (%) E3	persentase sel hidup (%) F1	persentase sel hidup (%) F2	persentase sel hidup (%) F3	persentase sel hidup (%) G1	persentase sel hidup (%) G2	persentase sel hidup (%) G3	persentase sel hidup (%) H1
0.371112	-0.17672	0.97196	-0.5655	0.653864	1.184025	13.67813	14.10226	16.15221	6.450283
5.849434	5.619698	5.72573	8.394204	8.924364	8.694628	17.1065	15.67507	16.13454	10.21442
9.472196	8.093779	12.90057	14.11993	15.63973	13.92554	52.87465	59.80207	71.65999	19.38619
60.36758	46.91918	65.26272	60.36758	60.79171	63.56621	62.57658	69.78676	72.95005	36.68709
69.89279	70.29925	73.62158	89.91517	81.75071	83.30584	84.43685	81.0615	78.23398	77.72149
77.68615	68.97385	82.08648	84.82564	93.82069	101.3313	93.34354	109.9376	93.20217	78.11027

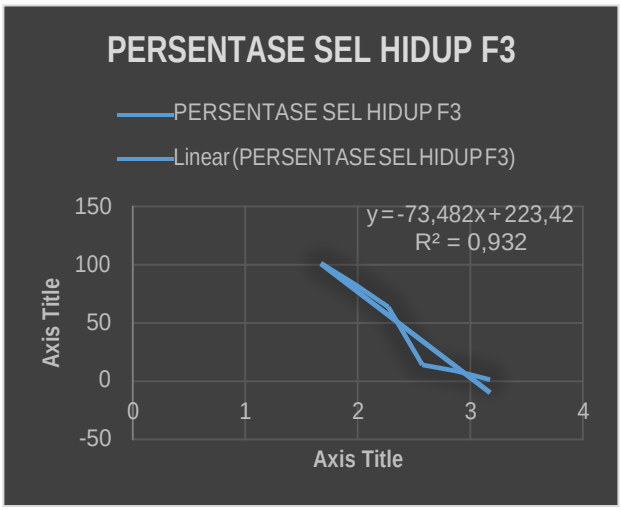


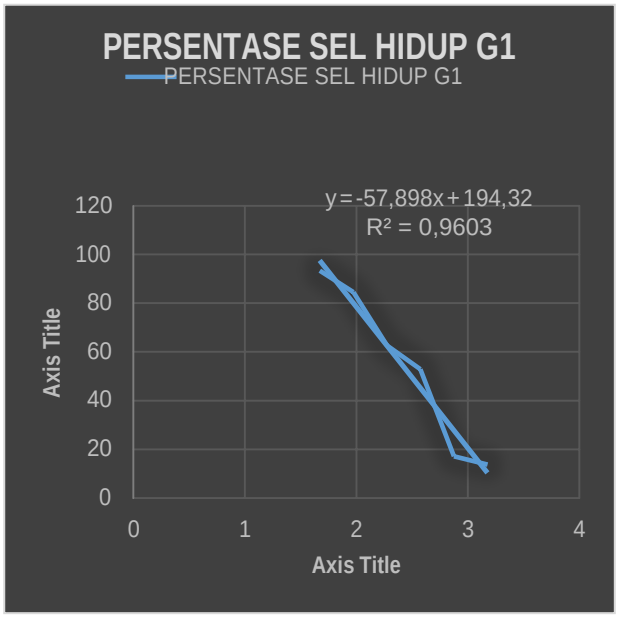


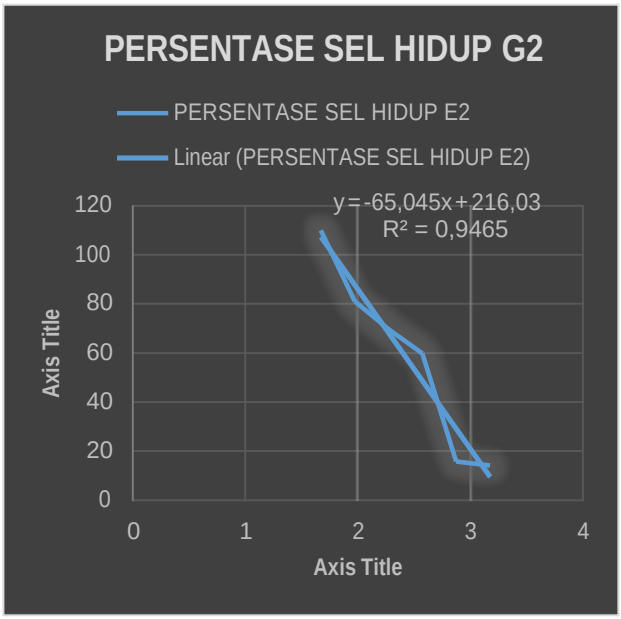


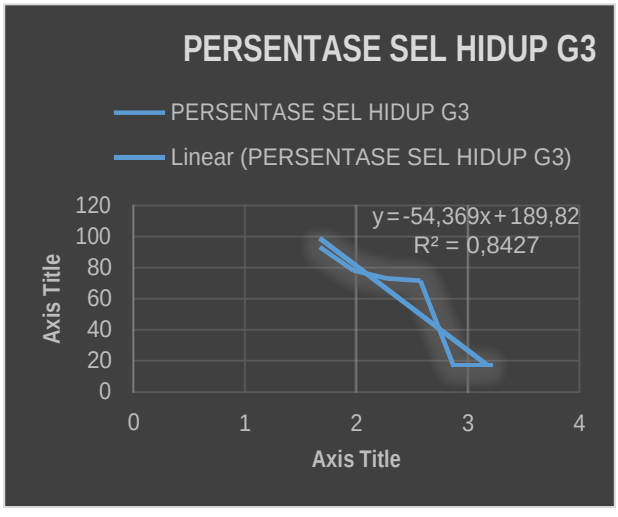


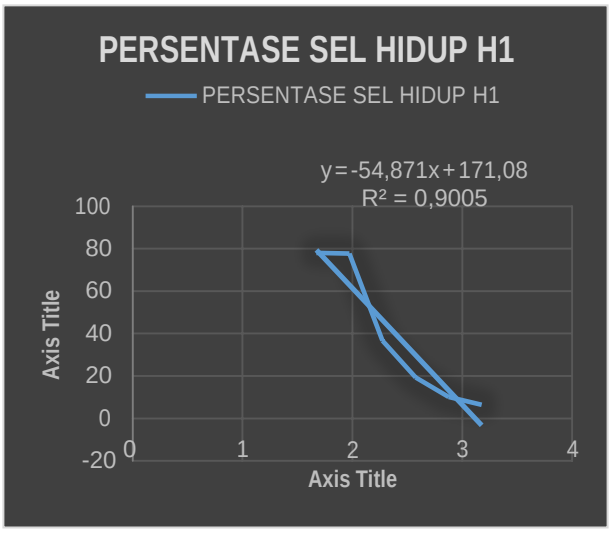

PERSENTASE SEL HIDUP F2
PERSENTASE SEL HIDUP F2
Linear (PERSENTASE SEL HIDUP F2)
y = -69,235x + 211,39
R² = 0,9383
Axis Title
Axis Title

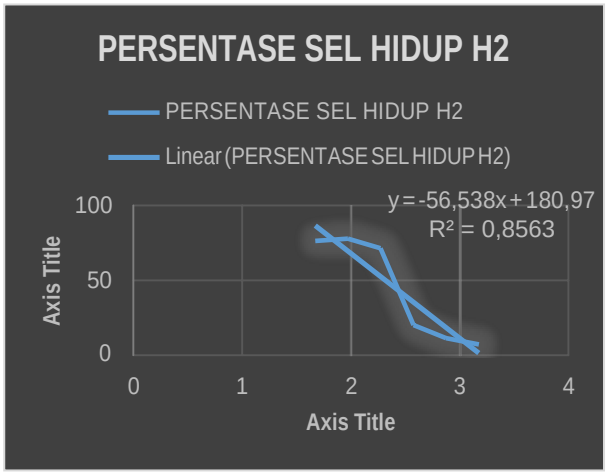

PERSENTASE SEL HIDUP F3
PERSENTASE SEL HIDUP F3
Linear (PERSENTASE SEL HIDUP F3)
y = -73,482x + 223,42
R² = 0,932
Axis Title
Axis Title

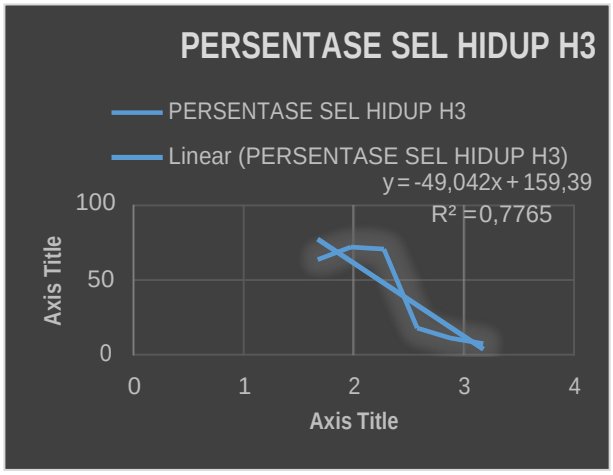

PERSENTASE SEL HIDUP G1
PERSENTASE SEL HIDUP G1
y = -57,898x + 194,32
R² = 0,9603
Axis Title
Axis Title


PERSENTASE SEL HIDUP G2
PERSENTASE SEL HIDUP E2
Linear (PERSENTASE SEL HIDUP E2)
y = -65,045x + 216,03
R² = 0,9465
Axis Title
Axis Title


PERSENTASE SEL HIDUP G3
PERSENTASE SEL HIDUP G3
Linear (PERSENTASE SEL HIDUP G3)
y = -54,369x + 189,82
R² = 0,8427
Axis Title
Axis Title


PERSENTASE SEL HIDUP H1
PERSENTASE SEL HIDUP H1
y = -54,871x + 171,08
R² = 0,9005
Axis Title
Axis Title



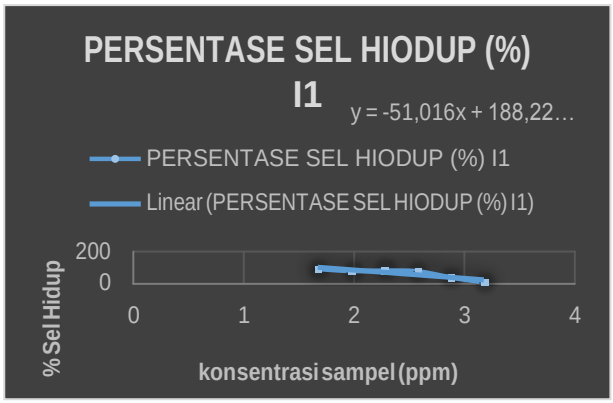


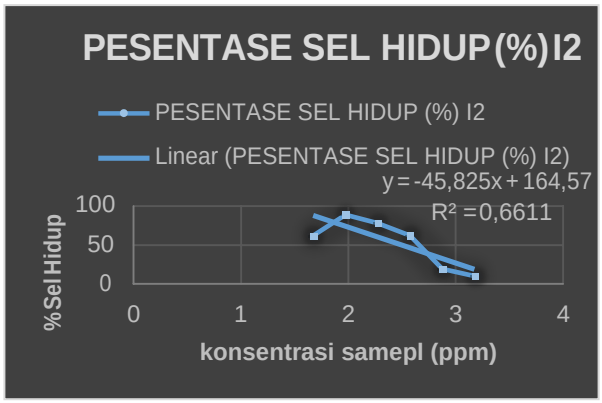
Absorbansi											
I			J			K			L		
0,161	0,1587	0,1617	0,1352	0,1334	0,1325	0,093	0,0932	0,0928	0,091	0,0903	
0,3315	0,2109	0,1882	0,1354	0,1303	0,1376	0,1363	0,1341	0,1346	0,1557	0,1578	
0,548	0,4527	0,4829	0,1801	0,1838	0,185	0,1824	0,1634	0,1722	0,4469	0,4382	
0,5681	0,5432	0,6029	0,4526	0,3688	0,4657	0,3932	0,4981	0,5332	0,4924	0,4956	
0,559	0,6036	0,642	0,5495	0,4755	0,5445	0,5639	0,5366	0,6016	0,5457	0,5087	
0,6288	0,4514	0,6119	0,4942	0,5593	0,5422	0,6344	0,5714	0,6128	0,5698	0,6525	

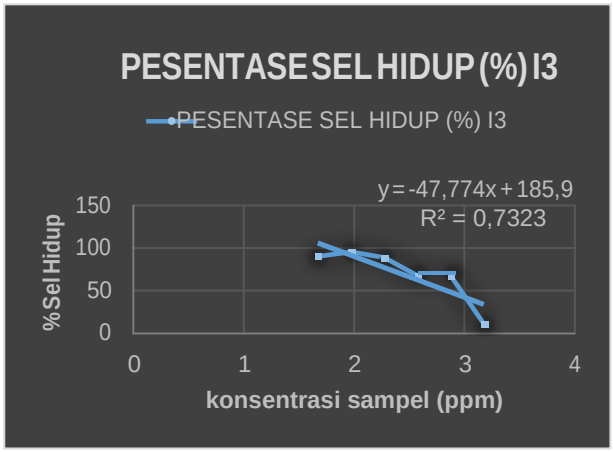
persentase sel hidup (%) I1	persentase sel hidup (%) 12	persentase sel hidup (%) I3	persentase sel hidup (%) J1	persentase sel hidup (%) J2	persentase sel hidup (%) J3
10,67389	10,26744	10,7976	6,114515	5,796418	5,63737
40,80467	19,49222	67,56008	6,149859	5,248586	6,538643
79,06456	62,22314	67,56008	14,04925	14,70311	14,91517
82,61664	78,21631	88,76649	62,20547	47,39632	64,5205
81,00848	88,8902	95,67625	79,32964	66,25236	78,44604
93,34354	61,9934	90,35697	69,55702	81,0615	78,03959

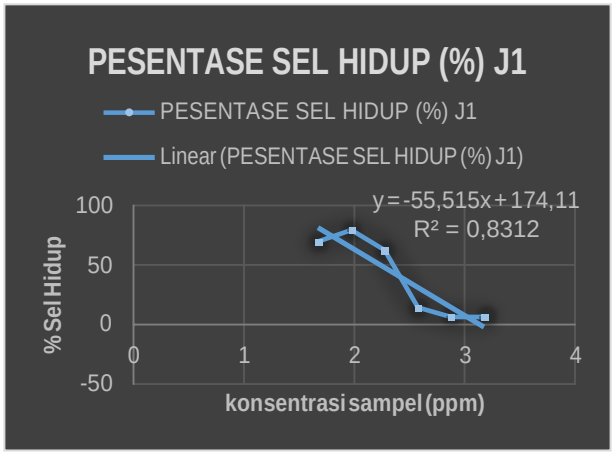
persentase sel hidup (%) K1	persentase sel hidup (%) K2	persentase sel hidup (%) K3	persentase sel hidup (%) L1	persentase sel hidup (%) L2	persentase sel hidup (%) L3
-1,34307	-1,30773	-1,37842	-1,69651	-1,82022	-1,71418
6,308907	5,920123	6,008483	9,737276	10,10839	9,24246
14,4557	11,09802	12,65316	61,19816	59,6607	59,05985
51,70829	70,24623	76,4491	69,23893	69,80443	76,71418

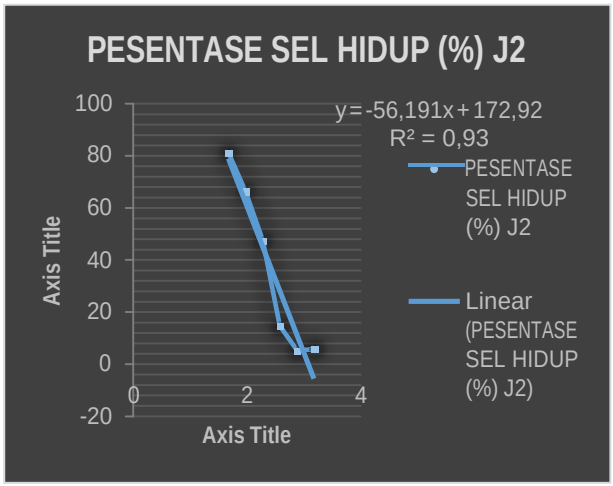
81,87441	77,04995	88,53676	78,65811	72,11946	89,98586
94,33318	83,19981	90,51602	82,91706	97,53181	95,37582

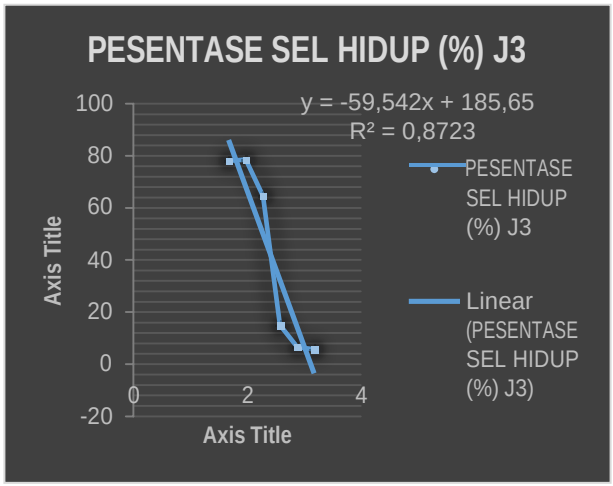


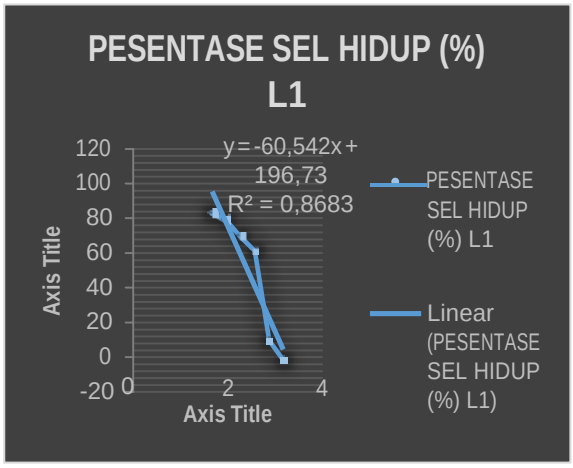


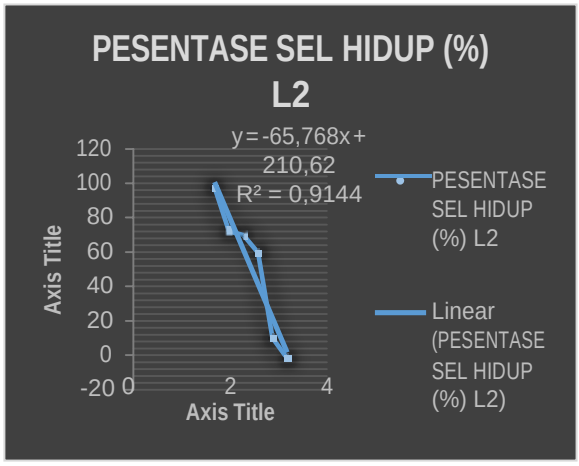


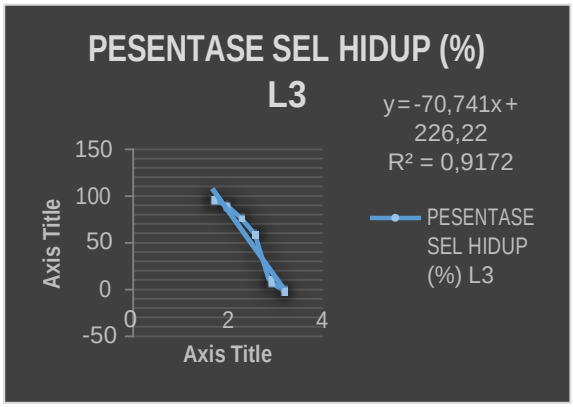






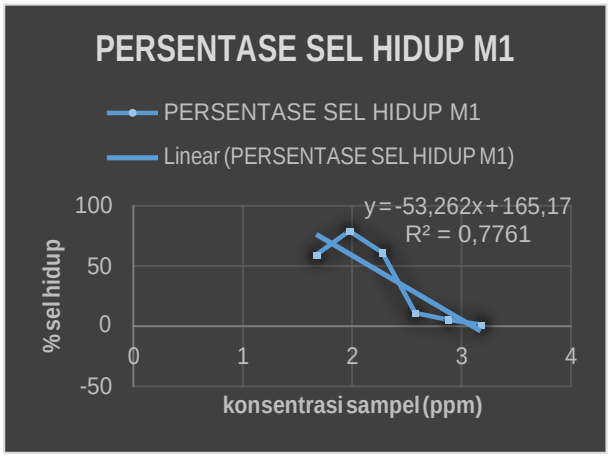


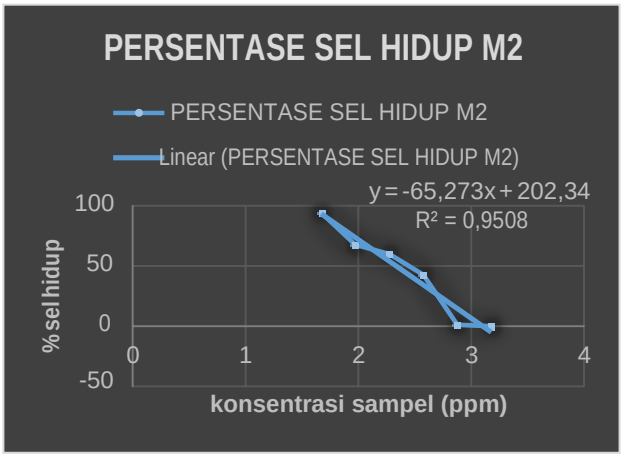


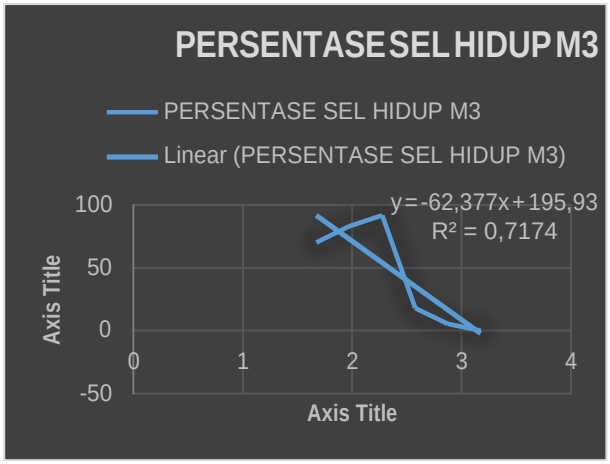


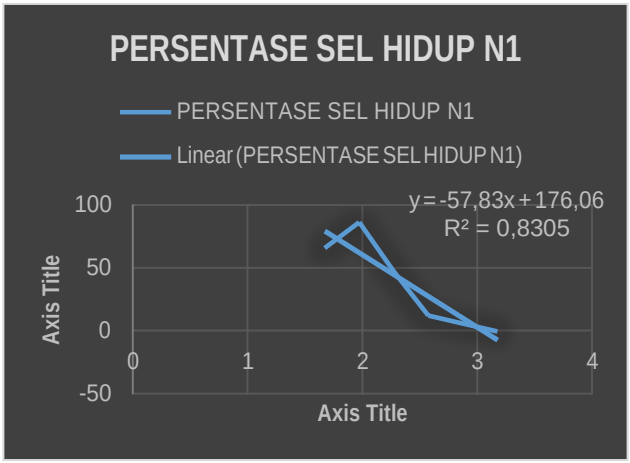
Absorbansi					
M			N		
0,1071	0,0999	0,1006	0,0967	0,097	0,101
0,1308	0,1066	0,1319	0,131	0,1131	0,1178
0,1617	0,341	0,2019	0,1682	0,2303	0,1928
0,4468	0,4396	0,6208	0,3662	0,3936	0,4129
0,5868	0,4821	0,5709	0,5886	0,4302	0,4754
0,4357	0,6332	0,4972	0,4721	0,6324	0,5837

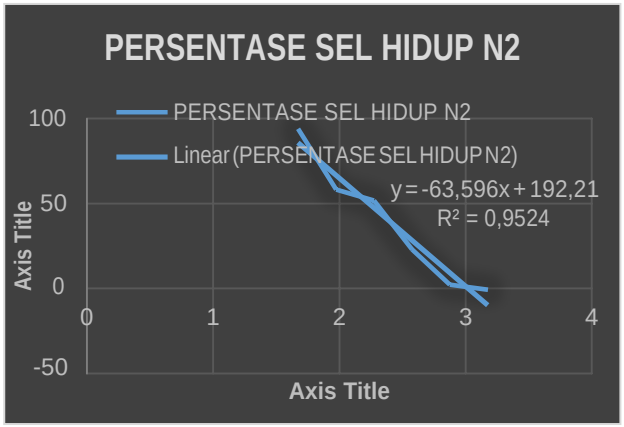
persentase sel hidup (%) M1	persentase sel hidup (%) M2	persentase sel hidup (%) M3	persentase sel hidup (%) N1	persentase sel hidup (%) N2	persentase sel hidup (%) N3
1,14868	-0,1237	-2,45248E-15	-0,68921	-0,63619	0,070688
5,336946	1,06032	5,53133836	5,37229	2,209001	3,039585
10,7976	42,48351	17,90174364	11,94628	22,92059	16,29359
61,18049	59,90811	91,92978322	46,93685	51,77898	55,18968
78,81715	67,41871	83,11145146	86,2394	58,24694	66,23468
59,2189	94,12111	70,0871819	65,65151	93,97974	85,37347

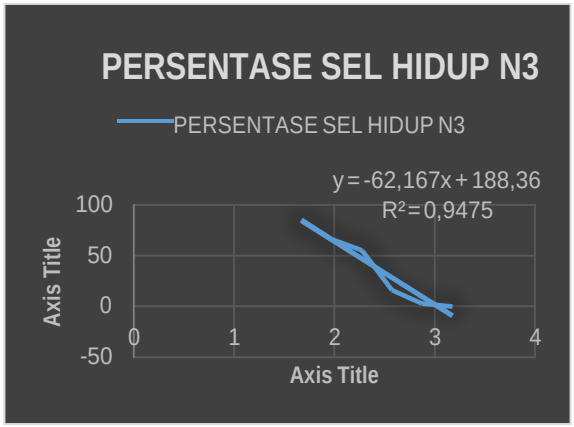












Tabel 3 kadar Absorbansi dan hasil perhitungan IC50 Ekstrak Tunggal

Absorbansi O (Tapak Liman)			
Kons (ppm)	Rep 1	Rep 2	Rep 3
1500	0,0729	0,076	0,0791
750	0,1435	0,1357	0,1467
375	0,1578	0,1813	0,1768
187,5	0,2463	0,2565	0,2618
93,75	0,359	0,3647	0,3697
46,875	0,3661	0,3829	0,396

Kons (ppm)	**% Sel Hidup O (Tapak Liman)**		
	Rep 1	Rep 2	Rep 3
1500	6,752603722	7,546525525	8,340447328
750	24,83353253	22,83592283	25,65306471
375	28,49581697	34,51425645	33,36178931
187,5	51,16100393	53,77326276	55,13061294
93,75	80,02390302	81,48369472	82,76421376
46,875	81,84224005	86,14478402	89,4997439

Absorbansi P (Kedelai)			
Kons (ppm)	Rep 1	Rep 2	Rep 3
1500	0,0882	0,0928	0,0851
750	0,2729	0,2639	0,2785
375	0,3484	0,3287	0,3421
187,5	0,3967	0,4003	0,376
93,75	0,402	0,4	0,3906
46,875	0,391	0,4155	0,3987

Kons (ppm)	% Sel Hidup P (Kedelai)		
	Rep 1	Rep 2	Rep 3
1500	10,67099	11,84907	9,877070172
750	57,97337	55,66843	59,40754653
375	77,3092	72,26396	75,69574868
187,5	89,67902	90,60099	84,37766775
93,75	91,03637	90,52416	88,11678334
46,875	88,21922	94,49377	90,19122418

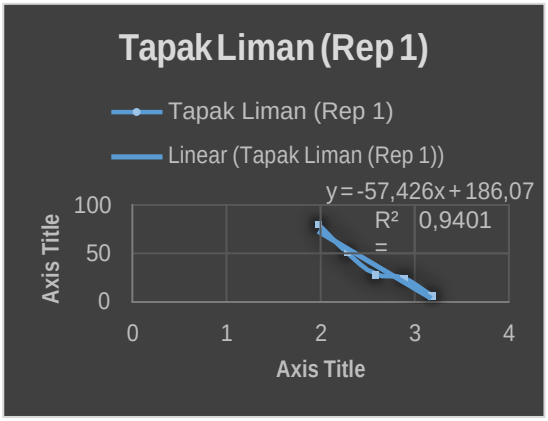
Absorbansi Q (Temulawak)			
Kons (ppm)	Rep 1	Rep 2	Rep 3
1500	0,0806	0,0915	0,0929
750	0,2856	0,2966	0,2964
375	0,3795	0,3648	0,3667
187,5	0,4153	0,3614	0,4203
93,75	0,3857	0,3966	0,413
46,875	0,416	0,4116	0,41

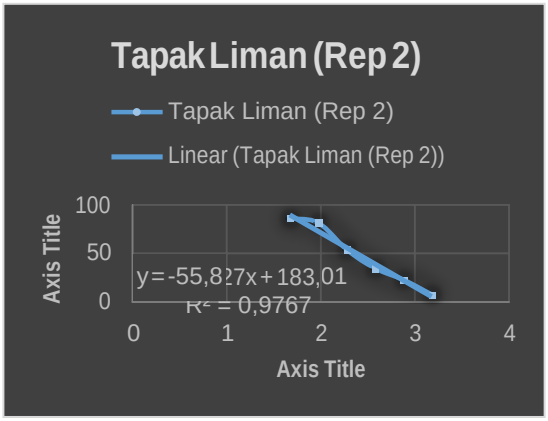
Kons (ppm)	% Sel Hidup Q (Temulawak)		
	Rep 1	Rep 2	Rep 3
1500	8,724603	11,51613	11,87467987
750	61,22588	64,04303	63,99180468
375	85,27403	81,50931	81,99590234
187,5	94,44255	80,63855	95,72306642
93,75	86,86187	89,65341	93,85350862
46,875	94,62182	93,49496	93,0851972

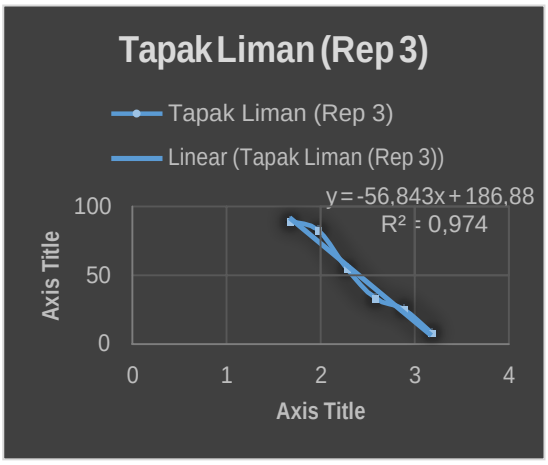
Absorbansi R (Obat)			
Kons (ppm)	Rep 1	Rep 2	Rep 3
150	0,0466	0,049	0,0504
125	0,0475	0,0544	0,0571
100	0,1742	0,159	0,1635
75	0,2509	0,2272	0,2168
50	0,2655	0,2371	0,2197
25	0,2373	0,2177	0,212

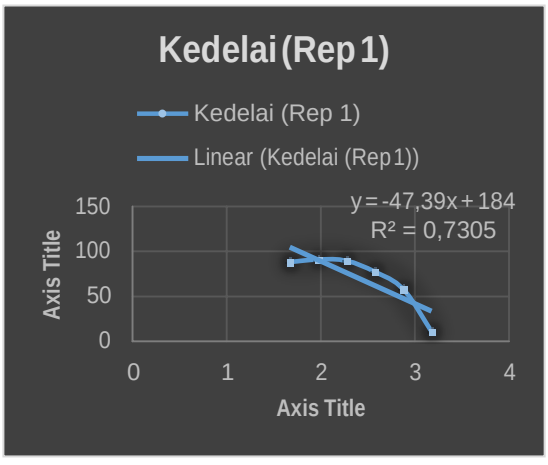
Kons (ppm)	% Sel Hidup R (Obat)		
	Rep 1	Rep 2	Rep 3
150	0,017074	0,631723	0,990268055
125	0,247567	2,014683	2,706163565
100	32,69592	28,80314	29,95560867
75	52,33908	46,26942	43,60594161
50	56,0782	48,80485	44,34864265
25	48,85607	43,83644	42,37664333

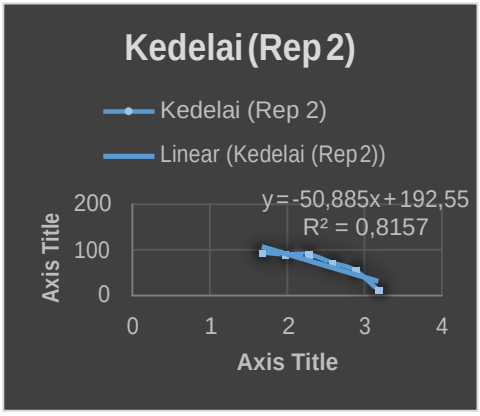
K. Sel	0,4355	0,4524	0,4231	**0,437**
K. Media	0,0458	0,0453	0,0485	**0,046533333**

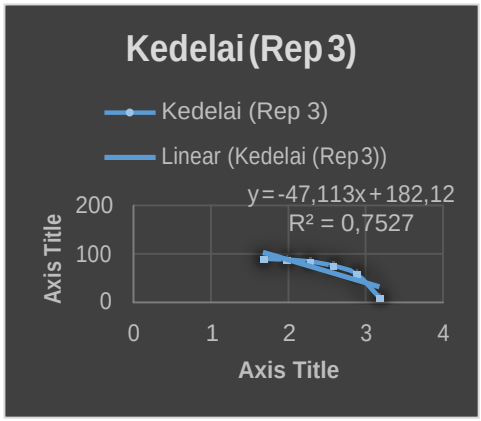


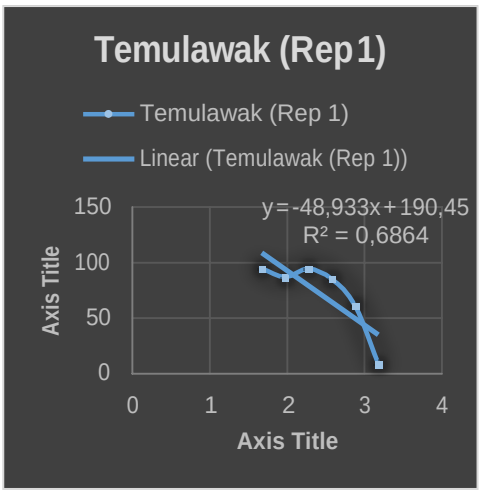


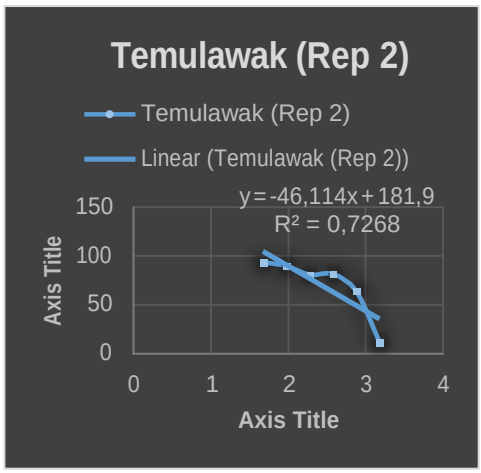


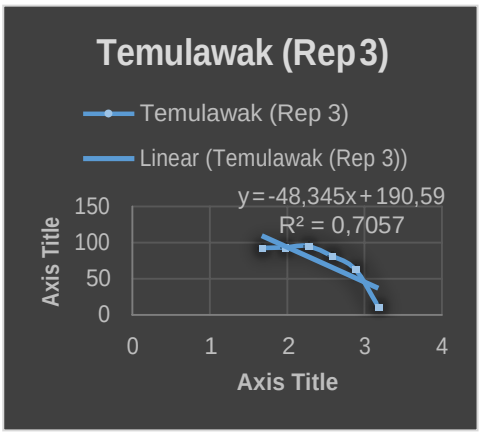


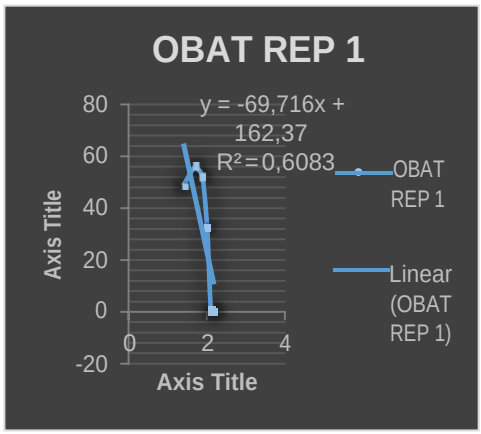

Kedelai (Rep 2)
Kedelai (Rep 2)
Linear (Kedelai (Rep 2))
y = -50,885x + 192,55
R² = 0,8157
Axis Title
Axis Title
200
100
0
0
1
2
3
4

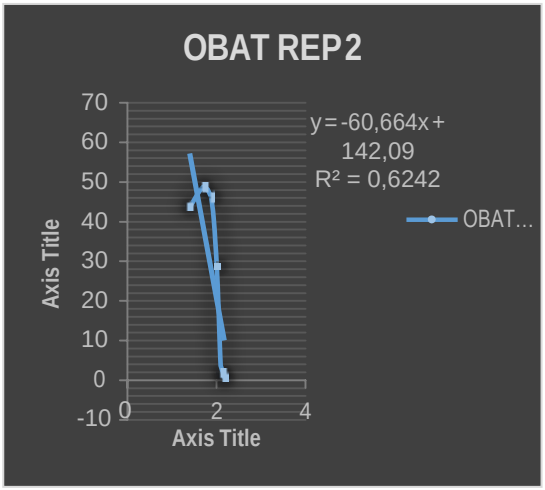

Kedelai (Rep 3)
Kedelai (Rep 3)
Linear (Kedelai (Rep 3))
y = -47,113x + 182,12
R² = 0,7527
Axis Title
Axis Title
200
100
0
0
1
2
3
4

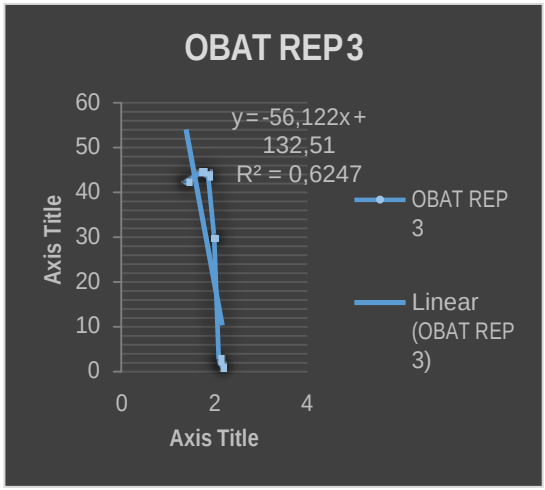

Temulawak (Rep 1)
Temulawak (Rep 1)
Linear (Temulawak (Rep 1))
y = -48,933x + 190,45
R² = 0,6864
Axis Title
Axis Title
150
100
50
0
0
1
2
3
4


Temulawak (Rep 2)
Temulawak (Rep 2)
Linear (Temulawak (Rep 2))
y = -46,114x + 181,9
R² = 0,7268
Axis Title
Axis Title
150
100
50
0
0
1
2
3
4

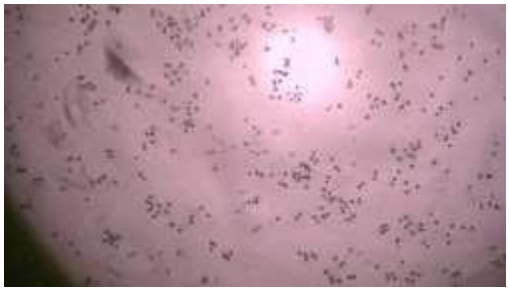

Temulawak (Rep 3)
Temulawak (Rep 3)
Linear (Temulawak (Rep 3))
y = -48,345x + 190,59
R² = 0,7057
Axis Title
Axis Title
150
100
50
0
0
1
2
3
4

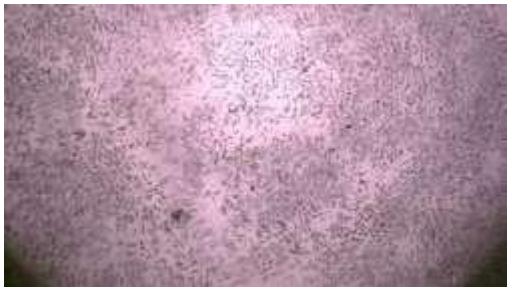

OBAT REP 1
y = -69,716x + 162,37
R² = 0,6083
OBAT REP 1
Linear (OBAT REP 1)
Axis Title
Axis Title
80
60
40
20
0
-20
0
2
4

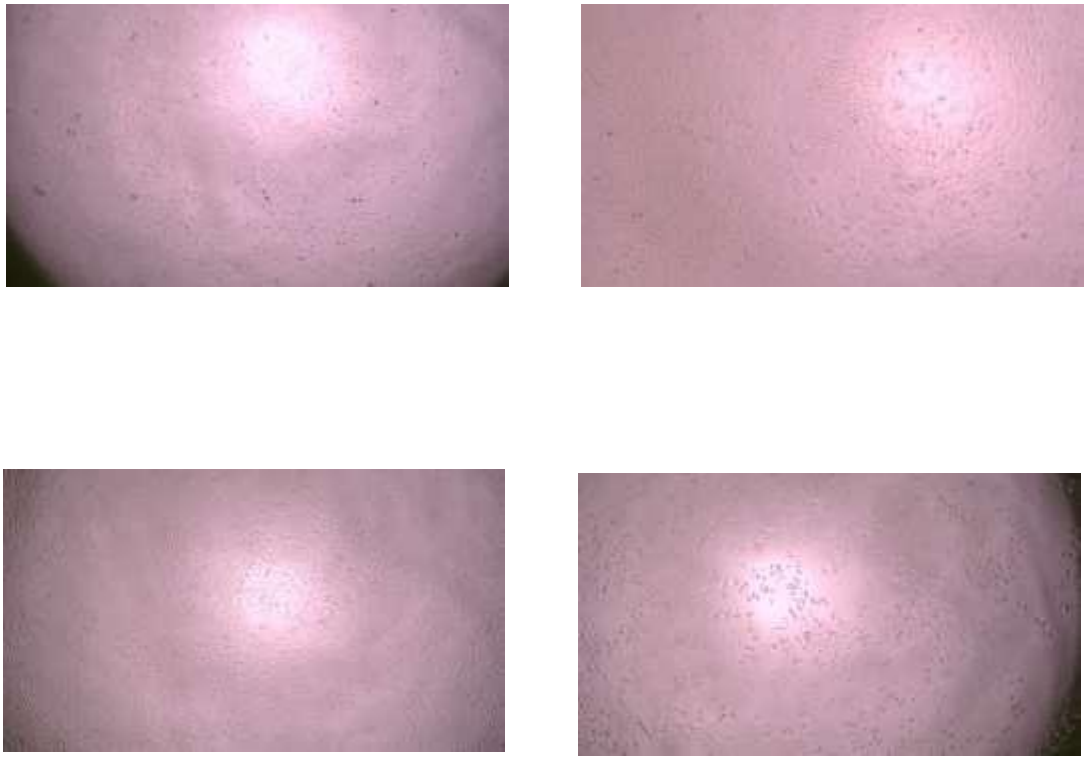




Selain dilakukan pembacaan menggunakan *ELISA reader*, profil sampel sel T47D juga dilihat di bawah mikroskop dengan hasil sebagai berikut :







Gambar 1.profil sampel sel T47D dibawah mikroskop

F. Pembuatan tablet

Tablet ekstrak daun tapak liman dibuat menggunakan metode granulasi basah dikarenakan selain tahan terhadap suhu pemanasan, ekstrak daun tapak liman memiliki sifat alir yang tidak baik sehingga penggunaan metode granulasi basah ini dapat memperbaiki sifat alir ekstrak daun tapak liman. Granul ekstrak daun tapak liman diperoleh dengan mencampurkan ekstrak daun tapak liman dengan bahan pengisi. Setelah homogen, ditambahkan dengan pengikat sampai diperoleh massa yang kempal. Cara mengetahuinya dengan menggunakan banana breaking test, penambahan bahan pengikat dilakukan sampai tidak ada lagi bagian campuran yang terjatuh. Campuran kemudian dibentuk menjadi granul menggunakan ayakan no. 12 dan hasilnya kemudian dikeringkan menggunakan oven dengan suhu 50^{o} c. Setelah kering granul diyak kembali menggunakan menggunakan ayakan no 12/30. Granul yang lolos ayakan 12 dan tidak lolos ayakan no 30 kemudian ditimbang dan ditambahkan bahan pelicin sebelum dicetak menjadi tablet. Penentuan formula tablet yang optimal ini menggunakan metode simplex lattice design yang mengoptimasi bahan pengisi dengan F1 (100% amylum), F2 (100% laktosa), dan F3 (50% amylum : 50% laktosa).

G. Uji sifat fisik tablet

Tablet kemudian diuji sifat fisiknya. Pengujian tersebut berupa uji keseragam bobot, uji kekerasan tablet, uji kerapuhan tablet, dan uji waktu hancur tablet.

Berikut ini adalah hasil pengujian sifat fisik tablet

Pengujian	F1	F2	F3
Keseragaman bobot	1,416 % ± 9,37	1,463% ± 9,54	2,11% ± 13,83
Kekerasan tablet	2,961 kg ± 0,419	10,1135 kg ± 0,356	3,982 kg ± 0,591
Kerapuhan tablet	0,572 % ± 0,042	0,225 ± 0,021	0,504 ± 0,171
Waktu hancur tablet	18 menit dan 31 detik (1111 detik)	11 menit dan 20 detik (680 detik)	13 menit dan 59 detik (839 tik)

Dengan menggunakan persamaan ( ) ( ) ( ), diperoleh nilai sebagai berikut:

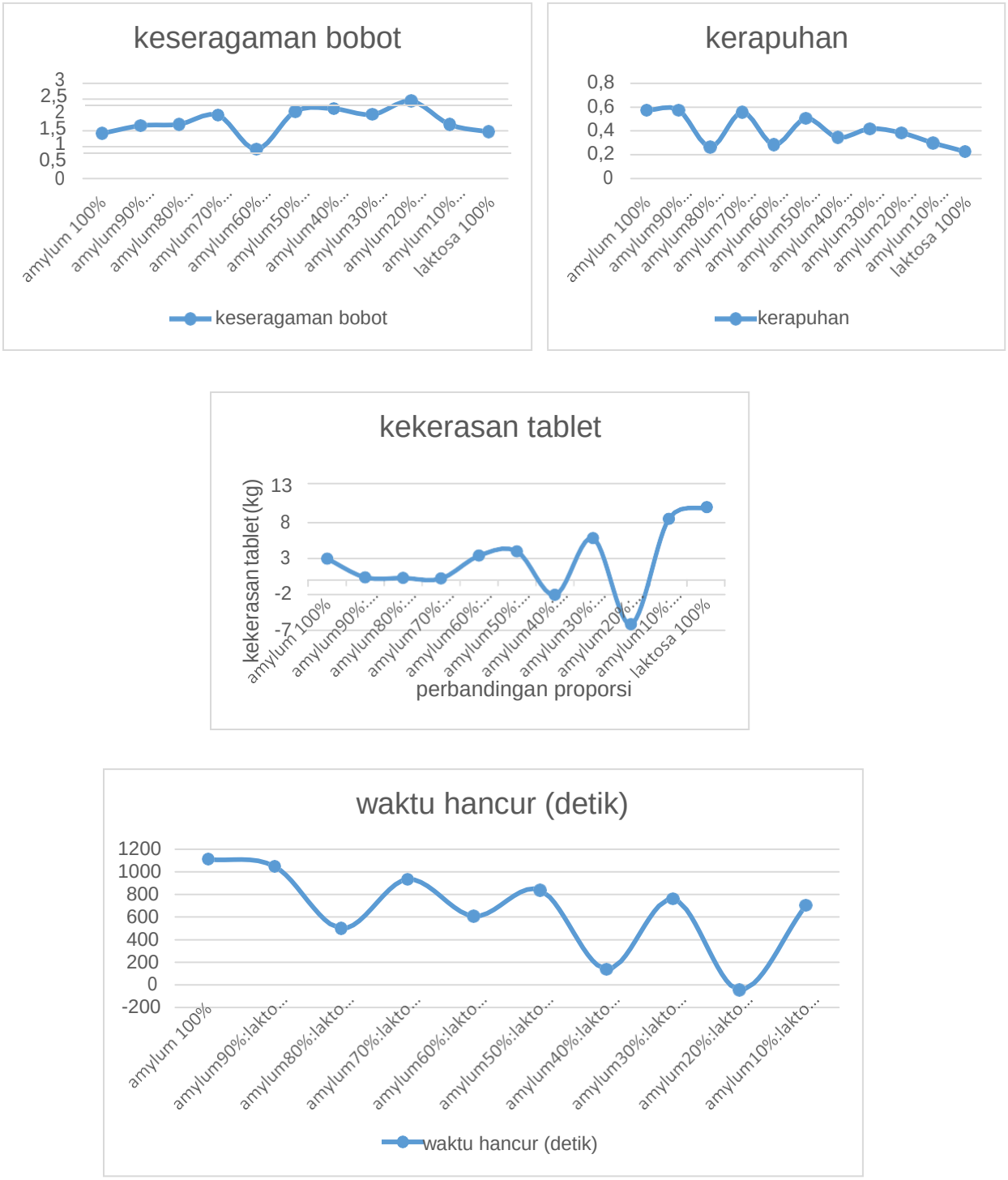
Pengujian	A	B	Ab
Keseragaman bobot	1,416	1,463	2,682
Kekerasan tablet	2,961	10,1135	-10,2208
Kerapuhan tablet	0,572	0,225	0,422
Waktu hancur tablet	1111	680	-226

Keterangan: Y = Respon (hasil percobaan)

(A)(B) = Kadar komponen dimana (A)+(B)=1

a,b,ab = Koefisien yang dapat dihitung dari hasil percobaan

Dari persamaan yang diperoleh, dapat dihitung besarnya respon (hasil penelitian) pada berbagai perbadingan proporsi lain yang ditunjukkan dengan grafik dibawah ini:



Gambar 2. Grafik respon perbandingan proporsi uji sifat fisik tablet

penentuan formula yang optimal

Setelah profil masing sifat fisik tablet didapatkan, maka selanjutnya dicari respon total yang merupakan penjumlahan dari respon-respon sifat granul masing-masing formula. Respon total dapa dihitung dengan rumus: r total = + + + ...

Nilai R merupakan respon yang diberi bobot 1. Dalam penelitian ini, digunakan empat respon respon yaitu keseragaman bobot, kekerasan tablet, waktu hancur tablet, dan kerapuhan tablet yang masing-masing diberi bobot 0,25.

Mengingat satuan masing-masing respon tidak sama, maka perlu dilakukan standarisasi penilaian respon dengan menggunakan respon berikut ini:

$$N = \frac{\quad}{\quad}$$

Keterangan:

X = respon yang diperoleh dari percobaan

Xmin = respon minimal yang diinginkan

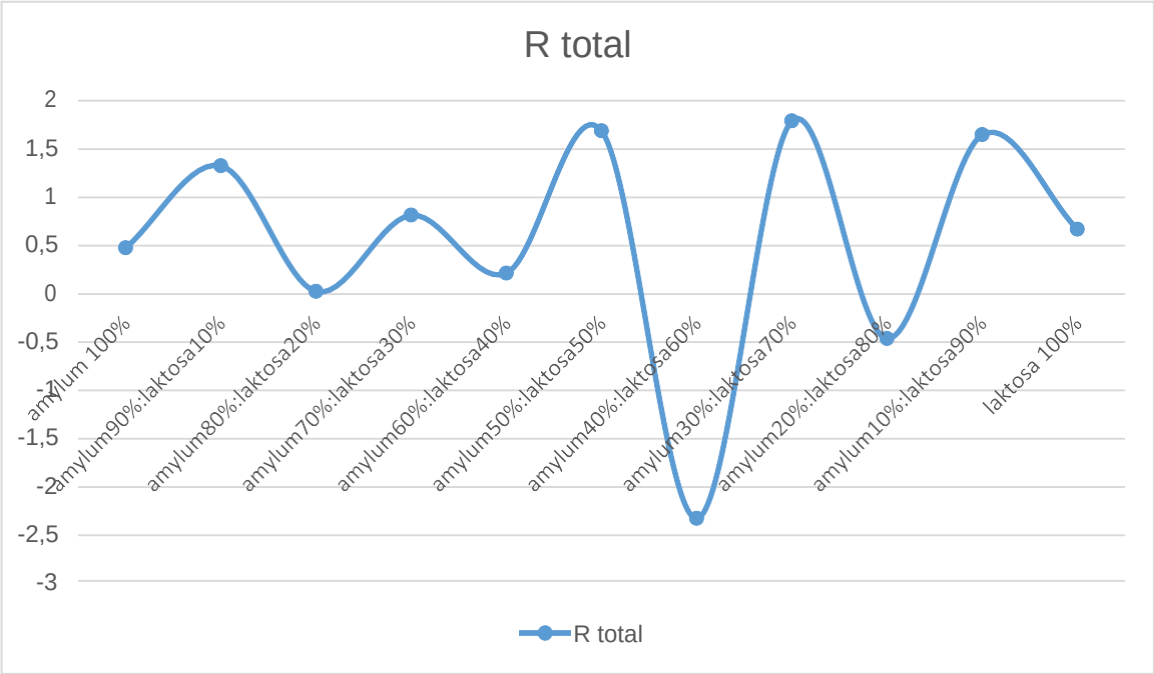
Xmax = respon maksimal yang diinginkan

jadi, R dapat dihitung dengan mengalikan N dengan bobot yang telah ditentukan.

Perhitungan respon total menjadi:

Rtotal = (bobot x n keseragaman bobot) + (bobot x n kekerasan tablet) + (bobot x n waktu hancur tablet) + (bobot x n kerapuhan teblet). Formula optimum yang dipilih ditentukan dengan melihat nilai respon total yang tinggi.

Berikut ini adalah grafik respon total uji sifat fisik tablet.



Gambar 3. Grafik respon total uji sifat fisik tablet

Berdasarkan grafik diatas, dapat dilihat bahwa perbandingan proporsi bahan tambahan mempengaruhi sifat fisik tablet. Dengan menggunakan metode simplex lattice design diperoleh formula optimum yang menggunakan bahan pengisi 30% amylum dan 70% laktosa. Formula optimum tablet ekstrak daun tapak liman adalah : zat aktif 248 mg; amylum 114,75 mg ; laktosa 267,75 mg; mg stearat 1%; talk 2%; sol. Gelatin 10% qs.

BAB VI
RENCANA TAHAPAN SELANJUTNYA

Setelah dilakukannya penelitian terhadap efektvitas tapak liman dan pengembangannya yang meliputi uji terhadap sel Hela, uji terhadap tikus, uji terhadap sel T47D, dan pengembangannya di bidang teknologi farmasi berupa optimasi pembuatan tablet, maka diharapkan di tahun selanjutnya formulasi tablet tersebut dapat dipatenkan dan digunakan untuk pengujian pra-klinik terhadap hewan uji, sehingga dapat diketahui jumlah zat aktif dalam formula tablet tersebut dapat memberikan efek maksimal dalam mencegah berkembangnya penyakit kanker.

BAB VII
KESIMPULAN DAN SARAN

Kesimpulan :

1. Senyawa aktif dari kombinasi tanaman tapak liman, kedelai dan temulawak dapat digunakan sebagai paliatif kanker payudara
2. Dengan menggunakan metode simplex lattice design diperoleh formula optimum yang menggunakan bahan pengisi 30% amylum dan 70% laktosa.

Saran :

1. Perlu dilakukan uji lebih lanjut untuk penelitian ini.
2. Melakukan optimasi menggunakan bahan tambahan yang lain dan pengujiannya.

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LAMPIRAN

Lampiran 1. Publikasi di Seminar Internasional

The cytotoxic effect of *Elephantopus scaber* Linn Extract Against Breast Cancer (T47D) Cells

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Abstract. Breast cancer is one of the main cause of death. *Elephantopus scaber* Linn (ES) which has been used as a traditional medicine contains an antitumor compounds. This study aimed to explore the active fraction from ethanolic extract of ES as anticancer and to determine its inhibition effect on the cell proliferation cycle of breast cancer (T47D) cells. The ES leaf was macerated with ethanol and then evaporated to get the concentrated extract. The extract was fractionated using petroleum ether, chloroform, and methanol respectively. The cytotoxic activity of each fraction was carried out with MTT method, and the inhibition of cell cycle test were observed by flowcytometry method. The result showed that ES and the fractions have cytotoxic activity against T47D cell lines with IC_{50} values of extract, petroleum ether, chloroform, and methanol fractions were 58.36±2.38, 132.17±9.69, 7.08±2.11, and 572.89±69.23 µg/mL. The inhibition effect of ethanol extract on the lifecycle of cells was occured in sub G1 phase. There was no prolonging of G1, S, G2/M and polyploidy phase of T47D cell lines. The chloroform fraction of ES is the most cytotoxic fraction against T47D cells without prolonging the cell lifecycle.

Keywords: *Elephantopus scaber*, cytotoxic, T47D, IC_{50}, cell cycle

1. Introduction

Breast cancer is the most common cancer among women and often causes death. The incidence of breast cancer is still high and difficult to be cured although the therapy has developed and progressed rapidly. The common treatment of breast cancer is consist of surgery, radiotherapy, chemotherapy and hormone therapy [1, 2].

Chemotherapy is a choise to stop the growth of cancer cell, but it is very toxic and has many side effects. The failure of chemotherapy can be associated with the failure of anticancer agents to induce programmed cell death (apoptosis). There are many reports of cancer cells resistance to chemotherapy. The resistance can be caused by overexpression of PGP in cells that lead to the presence of drug efflux out of the cell. Therefore, development of new cytotoxic agents for cancer therapy is urgently needed [3].

Many cytotoxic agents were provided in medicinal plants. *Elephantopus scaber* is a plant which has been reported as cytotoxic agent. The extract and fractions of *E. scaber* was proven to induce apoptosis against cervical cancer [4, 5]. *E. scaber* has been used traditionally to treat various deseases [6].

The anticancer potency of some chemotherapy was associated with the capability to inhibit the cells growth at certain phase of cell lifecycle. Based on differences in DNA content, cells can be distributed in phases of the cell cycle i.e. sub G1, G1, S, G2 and M as well as the polyploidy cells [7, 8]. The objective of this research was to explore the potency of ES extract as cytotoxic agent against breast cancer cells and screening the active fraction from the extract. The active fraction was then assayed the potency for inhibiting cell cycle.

2. Materials and Methods

a) Plant material collection

The plant material was collected from Merapi Farma, Yogyakarta, Indonesia. It was dried and blended.

b) Extraction and fractionation

The 200 g of ES powder was macerated using 900 mL of ethanol. The extract was collected and evaporated to get the concentrated extract. The concentrated extract was subsequently dissolved in petroleum ether and shaked for 6 hours and allowed to equilibrium for 24 hours. The soluble fraction was separated as petroleum ether fraction and the non soluble fraction was then fractionated using chloroform, ethyl acetate and methanol respectively. All fractions were evaporated and the dried fractions were collected.

c) Cytotoxicity test

Cytotoxicity assay was carried out using MTT method. The 100 µL suspension of T47D cells at a density of $1x10^4$ in 96 wells micro-plate were incubated for 24 h at 37°C (5% CO_2) with 100 µL RPMI medium and ES fraction with concentration series of test 2000; 1500; 1000; 800; 400; 200; 100; 50; 25; 12.5; 6:25; and 3.125 µg/mL.

After that, media was discarded by inverting the plate slowly ad followed by adding 100 □ L of MTT, then incubated for 4 hours at 37°C, 5% CO_2. After incubation, the mixture were added with 100 mL of SDS solution in 0.01 N HCl and incubated for over night at room temperature. The microplate were read by ELISA reader at 550 nm wavelength. The IC_{50} values were then calculated from percentage of living cells [9].

d) Flowcytometric analysis

The T47D cells was treated using ES extract with concentration equal to ½IC_{50} and IC_{50} and incubated for 24 hours. The cells was then washed using PBS and subsequently was added with 100 µL of Annexin V-PI and 350 µL of PBS. Furthermore, the cells was given DNase free RNase (20 µg/mL) and incubated at 37°C for 10 m. The cell was analysed using FACS Calibour Flowcytometry.

Results and Discussion

Medicinal plants produce a lot of bioactive compounds and can be used in treatment of many deseases. The exploration of therapeutic potential of the plants has been carried out since several decades ago. One of them is addressed to find the potential of anticancer. Cancer is one of the major health problems and lead to a high of death numbers. Breast cancer is the most common cancer among women. The great potential of plant-based compounds for the treatment and prevention of cancer is attributed to their safety, low cost, and oral bioavailability.

E. scaber have been used traditionally to treat various diseases as antiinflammation, diarrhea, hepatitis, arthritis. With the diverse traditional applications of *E. scaber*, United Nations Development Program has recommended *E. scaber* as a potential natural herb which should be further studied [6].

3.1. Cytotoxicity test

Fractionation process was conducted using petroleum ether, chloroform and methanol to separate the compounds with different polarity. It would be useful to get the fraction with the highest cytotoxicity. In this study, T47D cell lines were used as targetted cell in the cytotoxicity test of ES fractions. The T47D cell lines was derivated from breast cancer cells, so it can become the tested cells to find the potential activity of ES fraction against breast cancer cells. The citotoxic activity was evaluated by MTT method [10]. It was based on the activity of tetrazolium succinate reductase enzyme in viable

cells reducing tetrazolium salt *(3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazolium bromide)* to produce formazan crystal [9]. The concentration of dissolved formazan was measured by spectrophotometric method.

Figure 1 showed the percentage of T47D cell viability after ES fraction treatment. The viability of T47D cells were shown in dose dependent manner. The higher concentration resulted the more viability of the cells. The IC_{50} value of ES extract and each fraction were performed in Table 1.

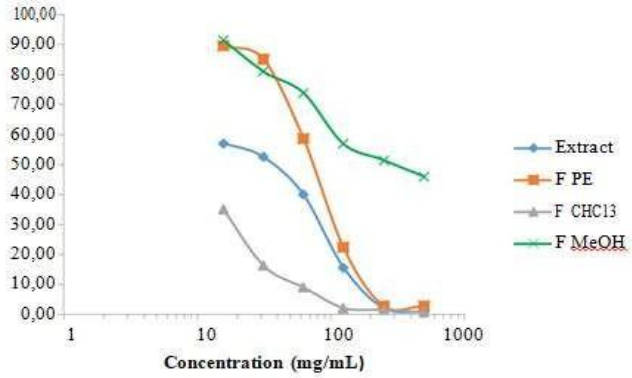

Figure 1. The cytotoxicity of ES extract and fractions against T47D cells.

Table 1. The cytotoxicity of ES extract and fraction against T47D cells presented as IC_{50}.

Fraction	IC_{50} (□g/mL)
Extract	58.36 ± 2.38
F PE	132.17 ± 9.69
F $CHCl_3$	7.08 ±2.11
F MeOH	572.89 ± 69.23

Based on the Table 1, it's revealed that chloroform fraction (F $CHCl_3$) treatment resulted the lowest IC_{50} indicated the high cytotoxicity against T47D cells. Some chemical constituent has been reported and identified as antitumor were deoxyelephantopin [11], scabertopinol, trans-caffeic acid, methyl 3,4-dicaffeoylquinate, luteolin-41-O-β-D-glucoside, trans-p-coumaric acid, indole-3-carbaldehyde, methyl trans-caffeate, luteolin-7-O- glucuronide 6"-methyl ester, and luteolin [12].

Figure 2 showed the cytotoxicity of the chloroform fraction. The morphology of cells in the control group (without ES fraction treatment) is very different from the treatment (using 1.5625 µg/mL of chloroform fraction) group. In the control group, the cells shape is tapering with nucleus looked clearly. On the other hand, the cells in the teatment seemed more little and circle with lower density than control

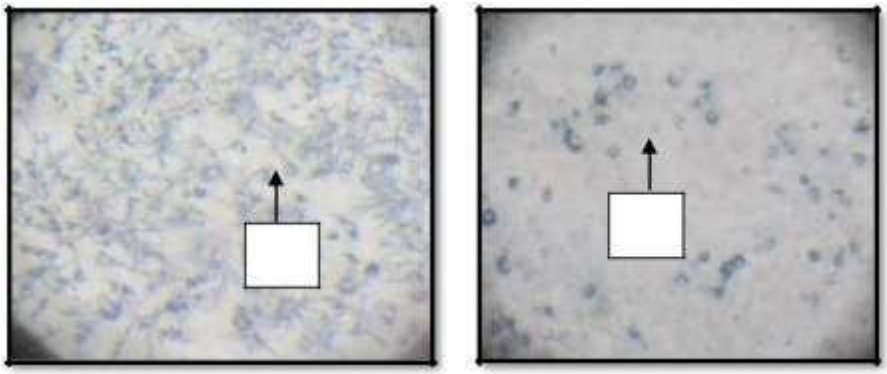

figure 2. Microscopic performance of T47-D cells of control group (A) and group of treatment with 1.5625 µg/mL of chloroform fraction (B)

3.2. Flowcytometric analysis3.2. Flowcytometric analysis

The flowcytometric analyses was subject to study the effect of ES extract to the cell cycle. The flowcytometry can inform the cells distribution in phases of sub G1, G1, S, G2/M, and the polyploidy cells based on the amount of chromosome set. Effect of the extract can be known by comparing the effect between control and treatment. The concentrations of ES extract used in this study were equal to ½ IC_{50} and IC_{50}.

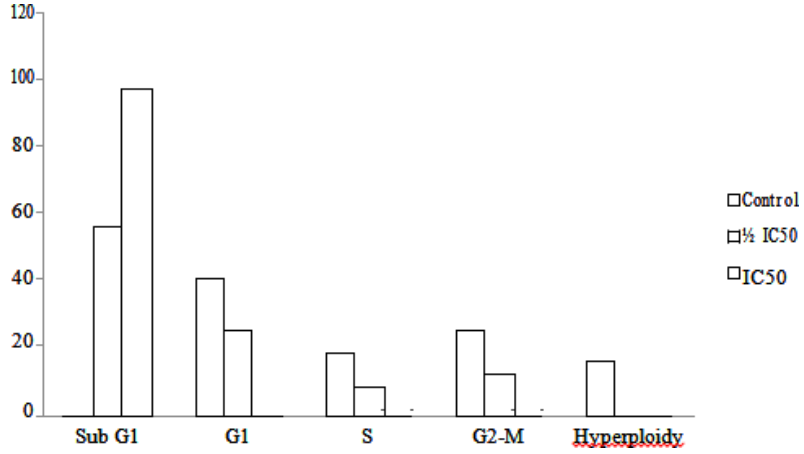

Figure 3. Comparation of T47D cell lines distribution in 5 cell phases among the control and the treatment with ½ IC_{50} as well as IC_{50} concentration of ES extract.

Figure 3 showed that in the control, the cells were distributed in all phases, mainly in G1 (41.07%), S (17.34%), and G2-M (25.34%) phases. The sub G1 phase only contained 2,27% of cells total. In the treatment of ½ IC_{50} of ES extract, the cells were most accumulated in Sub-G1 phase (55.34% of cells total). There were decending of cell concentration in phase of G1, S, G2-M and the polyploidy cells compared to the control. It means that this treatment resulted cell growth inhibition since the sub G1 phase or the cells were dead before growing to the next stage. Nevertheles, the other cells still develop in the next steps. The same result occured when the cells were treated by ES extract with the concentration equal to IC_{50}. Almost all of cells (96.95% of cells total) were detected in sub G1 phase. This ES extract concentration arrested all cells in sub G1. Accumulation of cells in sub G1 phase m

consist of apoptotic and/or necrotic cells [13–15]. Many factors may contribute the apoptosis as well as necrosis process. Hence, this study can be use to observe what mechanism inducing cells death in the next study.

Conclusions

E. scaber extract exerted anti-cancer activity on breast cancer (T47D) by cell cycle arrest in sub G1 phase. The chloroform fraction of *E. scaber* ethanolic extract was the highest active fraction.

Acknowledgement

The authors thank to Ministry of Research, Technology and Higher Education for funding this research. We also thank to Bariadi Nugroho, Panji Ratih Suci, Emi Syafaati, Kuntum Zahro Wardati, Irma Sari Tilawah and Muhammad Lukman for assisting during research.

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Elephantopus scaber Linn Extract Induces Apoptosis and Activates Caspase Cascade in T47D Cancer Cell Line

Abstract

Elephantopus scaber Linn. was used traditionally for treating various diseases. Previous studies found the cytotoxicity of this herb against different cell lines. This study aimed to determine the effectiveness of the ethanolic extract of *E. scaber* in inducing apoptosis by observing its effect on caspase cascade. This ethanolic extract was obtained by maceration method using 96% ethanol. It was fractionated with petroleum ether to discard compounds with very low polarity. This step was followed by the fractionation with chloroform to isolate compounds with optimum polarity. The soluble part in chloroform was dried and used for the assay. The immunocytochemistry method used specific antibodies for caspase-8, caspase-3, and caspase-9 to observe the expressions of each caspase. T47D cell line was treated with the chloroform fraction of *E. scaber* with the concentrations of 7.06 µg/ml and 3.53µg/ml. The results of the immunocytochemistry showed that this chloroform fraction increased the expressions of caspase-8, caspase-3, and caspase-9 proteins significantly. Such increase led to apoptotic cells. This finding supported the development of *E. scaber* extract as an anticancer agent.

Keywords: *Elephantopus scaber*, apoptosis, caspase-8, caspase-3, caspase-9, immunocytochemistry

Introduction

Cancer is a major health problem that causes death after cardiovascular disease. Breast cancer is the first ranked most common malignancy in female population [1]. Although cancer treatment has currently used different methods, it still does not provide any satisfying results. Also, cancer therapy has many side effects and damages the normal cells.

Cancer progresses due to an imbalance between cell proliferation and cell death. The process of programmed cell death, or apoptosis, is considered vital in normal homeostatic settings. This process produces a balance in the number of cells by eliminating damaged cells and physiological proliferation. The defects in the mechanisms of apoptosis play essential roles in tumor development as they allow neoplastic cells to survive and reproduce uncontrollably[2]

Elephantopus scaber L. has been used traditionally to treat various disease. It has been reported to have a cytotoxic effect and induce the apoptotic death of HeLa cancer cells

Some of its active compounds have been successfully isolated, particularly deoxyelephantopin and isodeoxyelephantopin of the sesquiterpene lactone class [3]. Deoxyelephantopin inhibits the growth of cancer cells by an apoptotic mechanism in which the caspase cascade, i.e., caspases-8, -9, -3, and -7, are activated [4]. This finding affirms the potential of *E. scaber* L.as an anticancer [7]. The fractions obtained from the extract of *E. scaber* L. are empirically found to exhibit cytotoxic activities, which are indicated by IC_{50} value. The chloroform fraction of the leaves of *E. scaber* L. shows cytotoxic activity against T47D breast cancer cell line with IC_{50} of 7.06 ug/ml [6]. The understanding of the mechanism by which a compound works is fundamental to drug development. This study aimed to identify the efficacy of the active fraction of the ethanolic extract of *E. scaber* L. in inducing apoptosis by the activation of caspase cascade.

Materials and Methods

.*Materials*

The leaves of *E. scaber* L. were obtained in Yogyakarta, Indonesia. The plant was identified as *Elephantopus scaber* Linn. from the family Compositae in Laboratory of Biology, Universitas Ahmad Dahlan under the supervision of Assoc. Prof. Hadi Sasongko, and the number of the herbarium specimen is 073/Lab.Bio/B/VII/2016.

.*Extraction and Fractionation*

The leaves of *E. scaber* L. were dried in an oven at 50^{o}C. The dried leaves were powdered and sieved with 20/40 mesh. The powder that passed through the 20 mesh but was retained by the 40 mesh was used for extraction. The extraction was performed by maceration with 96% ethanol solvent. The maceration was replicated three times to maximize the collection of the compounds. The macerate was evaporated with a vacuum evaporator to obtain a viscous extract.

The viscous extract of 20 grams of *E. scaber* L. was dissolved and shaken in 100 ml of petroleum ether to discard compounds with very low polarity. The soluble fraction of the petroleum ether was separated from the insoluble matter, which was later dissolved in 100 ml of chlorofor. The chloroform fraction was evaporated until a solid chloroform fraction was

formed. The fractionation of the extract with chloroform was performed three times to get the maximum active fraction.

Sample Preparation

A sample of 10 mg was dissolved in 1 ml of dimethyl sulfoxide (DMSO). Then, it was diluted with RPMI to achieve the concentrations of 7.06 µg/ml and 3.53 µg/ml by gradual dilution. The treatment used these two concentrations because the IC_{50} of chloroform fraction was 7.06 ug/ml [8]. At this concentration, the cell growth and protein expression were easily observable due to the presence of adequate viable cells. The final concentration of DMSO in the sample was 0.007%. Exposure to 1% DMSO is empirically proven to inhibit cell survival insignificantly [9]. Therefore, this concentration is not toxic to cell growth.

Immunocytochemistry

The expressions of the caspases were observed with immunocytochemistry technique [10]. T47D cells were grown in a 24-well microplate. They were left to attach and grow in the bottom of the plate after overnight incubation. The microplate was taken from the incubator, and the culture medium was then removed from each well using a micropipette. A solution of 1 ml were sampled from extract with concentrations of 7.06 µg/ml and 3.53 µg/ml, transferred into the well, and then incubated for 24 hours. After the incubation, all culture mediums were removed from the well, added with 300 µl of PBS, and then left for 5 minutes. The PBS solution was discarded. The culture mediums were added with 300 µl of distilled water, left for 5 minutes, and then discarded. The cells were fixed with 300 µl of methanol and left for 10 minutes before the methanol was discarded. After the fixation, the cells were washed two times with 300 µl PBS, added with 100 µl of hydrogen peroxide solution, and left for 5-10 minutes. The solution was removed and washed with 300 µl of PBS two times. Afterward, the cells were added with 100 µl of prediluted blocking serum and left for 10-15 minutes. They were then removed, added with 100 µl of primary anti-caspase-8, anti-caspase-3, and anti-caspase-9, and incubated for 24 hours. After the incubation, they were washed two times using 300 µl of PBS, added with 100 µl of secondary antibodies, and left for 20 minutes. Afterward, the cells were washed two times using 300 µl of PBS, added with 100 µl of HRP solution, left for 10 minutes, and then washed with PBS.

DAB solution were added to the cells and left for 2 minutes. After washing them with distilled water, the microplate was added with Mayer Hematoxylin solution and left for 5 minutes. The last step was washing the cells with 500 µl of distilled water and left them to

dry. The expressions of caspase-8, caspase-3, and caspase-9 were observed under a light microscope.

Analysis

Cells were observed under a light microscope with 100x magnification. The expressions of caspase 8, caspase-3, and caspase-9 were characterized by the color of the cell. The positive caspases appear in brown or dark color, while the negative ones have blue or purple color. The expressions were observed on six fields of view for every sample and presented as the percentage of positive expression compared to the total area of the cells.

Results and Discussion

.Extraction and Fractionation

The extraction of the leaves of *E. scaber* L. using 96% ethanol produced a concentrated extract with a dark color. The yield of the extraction was 8.5%, which was in line with the standard, i.e., higher than 2.7% [11]. Fat and other compounds with very low polarity were then removed with petroleum ether. Fractionation with chloroform aimed to isolate active compounds with optimum polarity. The result was 20.5% compared to the crude extract. The high cytotoxicity of chloroform fraction was indicated by IC_{50} of 7.06 µg/ml [6].

The Increased Expression of Caspase-8 by the Elephantopus scaber Extract

The activation of apoptosis-signaling pathways by anticancer drugs is frequently formed during the activation of caspases, a family of cysteine proteases that act as common death-effector molecules. Caspases can trigger apoptosis by cleaving various cytoplasmic or nuclear substrates, which are the morphologic features of apoptotic cells. The activation of caspase can be initiated in the plasma membrane with different mechanisms, by either death receptor-mediated signaling (receptor pathway) or mitochondrial pathway [12].

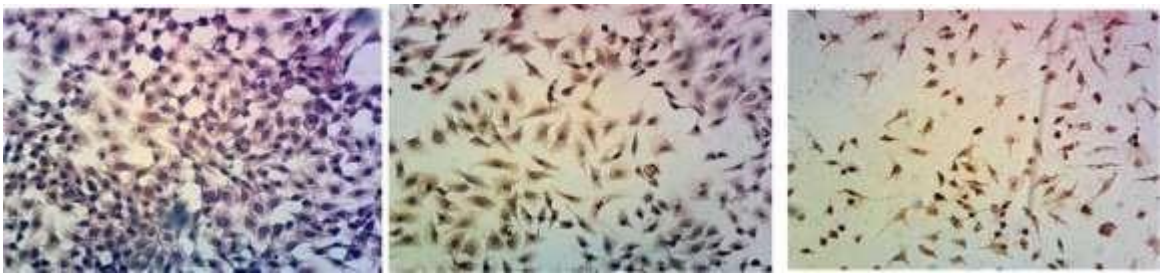
Elephantopus scaber was reported as a promising anticancer agent. Some active compounds that are isolated from *E. scaber* exhibit cytotoxic activity against some cell lines [4,13]. This study observed the expression of caspases after the addition of the fraction of *E. scaber* as a potential treatment for cancer. The expression of caspases involved a series of complex processes and many factors. The gene expression system, including initiation, transcription, translation, and other concomitant processes, was carefully controlled.

The expression of caspase-8 in T47D breast cancer cell line after treatment with *E. scaber* extract is presented in Figure 1. Treatment with 3.53 µg/ml increased the expression

of caspase-8 (Figure 1B). The dark brown cells were identified in nearly all culture cells. This expression was significantly different from the morphology of the control sample (Figure 1A). The dark brown color indicates high expression of caspase-8 following the treatment with *E. scaber*. A higher dose of the fraction of *E. scaber* (7.06 µg/ml) resulted in damaged cells (Figure 1C). Subsequently, the T47D cells entered the late stage of apoptosis and, then, necrosis. The morphology of the T47D cells was characterized by cellular shrinkage and apoptotic bodies in their surrounding. The morphological changes, signifying apoptosis, were observed in most cell types. These changes started with a reduction in cell volume and followed by the condensation of the nucleus [14].

At a concentration of 3.53 µg/ml (Figure 1B), treatment with *E. scaber* produced brown cells, indicating the expression of caspase-8 in the cytoplasm. This expression led to apoptotic cells. The calculation of the expression is summarized in Table 1.

The activation of caspase-8 after the treatment indicates the activation of the extrinsic pathway. Caspase-8 also has a significant role in the transcription of p53 tumor suppressor protein [15].



A **B**

C

Figure 1. The expressions of caspase-8 in T47D cells after treatment with *E. scaber*: (A) Control cells, (B) 3.53 ug/ml, and (C) 7.06 ug/ml.

Table 1. The calculation of the expression of caspase-8 in T47D cells after treatment with *Elephantopus scaber*

Treatments	The Expressions of Caspase-8 (% ± SD)

Control	0 ± 0%
3.53 µg/ml	96.62 ± 2.69%
7.06 µg/ml	100 ± 0%

The Increased Expression of Caspase-9

Caspase-9 is an initiator caspase that regulates the occurrence of apoptotic processes through the internal pathway. It is activated by binding cytochrome c to Apaf-1, which forms a complex known as apoptosome. This complex activates the caspase-9 zymogen (pro-caspase-9). Once activated, caspase-9 will trigger the activation of the effector caspase and cause apoptosis [16].

This research showed that the treatment of T47D cells with *E.scaber* increased the expression of caspase-9 (Figure 2). The calculation of the expression of caspase-9 after the treatment is presented in Table 2.

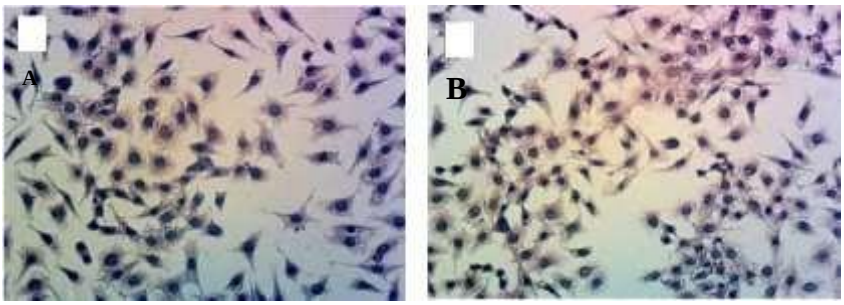


Figure 2. The expression of caspase-9 after treatment with *Elephantopus scaber*: (A) control and (B) 3.53 µg/ml

Table 2. The calculation of the expression of caspase-9 in T47D cells after treatment with *Elephantopus scaber*

Treatments	The Expressions of Caspase-9 (% ± SD)
Control	18.89 % ± 0.03
3.53 µg/ml	45.23 % ± 0.02

This study found that the expression of caspase-9 significantly increased after treatment with 3.53 µg/ml of *E. scaber* extract. In a normal condition (the control sample), the expression of caspase-9 in T47D cell lines was low (Figure 2A). After the treatment, it started to increase, proving the role of *E. scaber* in inducing of apoptosis through

The Increased Expression of Caspase-3

Caspase-3 is a proapoptotic agent that acts as a major effector caspase (executioner) in the process of apoptosis. It plays an essential role in breaking the apoptotic substrate and activating other effector caspases, including caspase-6 and caspase-7 [17]. Treatment with *E. scaber* extract was able to increase the expression of caspase-3, as presented in Figure 3 and Table 3.

During the research, caspase-3 seemed to be downstream of caspase-8. The increased expression of caspase-3 after treatment with *E.scaber* was most likely caused by the increased expression of caspase-8. Caspase-8 is an initiator caspase that cleaves pro-caspase-3 into activated caspase-3. Upon the activation, caspase-3 becomes capable of cleaving many cellular substrates and induces morphological changes like chromatin condensation, membrane blebbing and DNA fragmentation, indicating the process of apoptosis [18].

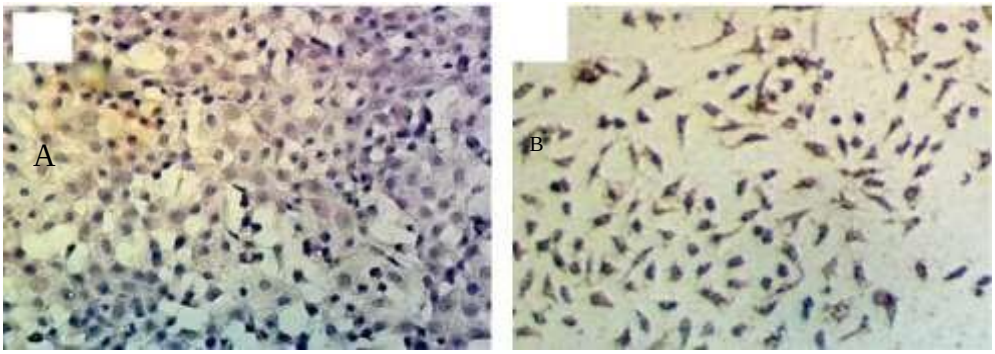


Figure 3. The expression of caspase-3 after treatment with *Elephantopus scaber*: (A) control and (B) 3.53 µg/ml

Tabel 3. The expression of caspase-3 in T47D cells after treatment with the extract of *Elepahantopus scaber*

Treatments	The expressions of caspase-3 (% ± SD)
Control	2.13 ± 0.028
7.06 µg/ml	17.65 ± 0.018

3.5.Elephantopus scaber-induced caspase cascade

The activation of caspase-3 involved the intrinsic and extrinsic pathways. Caspase-3 was activated via the extrinsic pathway (death ligand) where the death signal caused by the compound of *E. scaber* bound to the death receptor. This bond formed a trimer with FADD (Fas-Associated Death Domain) and activated pro-caspase-8. The active caspase-8 activated caspase-3 as an effector caspase.

Meanwhile, in the intrinsic pathway (mitochondria), treatment with E. *scaber* extract induced the release of cytochrome c, which later formed a complex with Apaf-1 and pro-caspase-9 known as apoptosome. The active caspase-9 activated caspase-3 as an effector caspase. This study found that after the treatment with *E. scaber* extract, the expressions of caspase-8, caspase-9, and caspase-3 were increased, suggesting that this extract induces the apoptosis through intrinsic and extrinsic pathways.

The results of this study were in line with previous research, which reported that caspase-3 induced apoptosis and mediated cell cycle arrest in T47D cells by isodeoxyelephantopin. *E. scaber* was also reported to induce cell cycle arrest at G2/M phase [4].

This study proved the potential of *E. scaber* as an anticancer agent. The extract of *E. scaber* exhibits cytotoxicity against various cancer cell lines, including MCF-7 breast cancer cell lines [4,13], A549 lung carcinoma cells [4], Hela cervical cancer cell lines [19], HCT human colon cancer cell lines, and Daltons Lymphoma Ascites (DLA) tumor cells [3].

This study also confirmed that the mode of death induced by *E. scaber* was apoptosis. The ability to induce apoptosis is an essential requisite of anticancer agents, including chemotherapeutic agents, hormones, and various biological compounds [16]. This study found that *E. scaber* induced the apoptosis of T47D breast cancer cell lines by activating caspase cascade. The expression of caspase-9, caspase-8, and caspase-3 increased significantly. Therefore, *E. scaber* is potentially developed as an anticancer agent.

Acknowledgment

This research was supported by the Indonesian Ministry of Research, Technology, and Higher Education as a part of the Fundamental Research Scheme.

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Ethanolic extract of *Elephantopus scaber* Linn effect on p53 dan Bcl-2 gene expression in rat breast cancer

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ABSTRACT

Expression of p53 induces tumor suppression in human cancer. In contrast, Bcl-2 expression is the most parameter used in breast cancer study to find out an anticancer. *Elephantopus scaber* L is one of plant having a potency as anticancer. This study aims to know the effect of *E. scaber* leaf extract on p53 and Bcl-2 gene expression in rat breast cancer. The rats were divided into five groups i.e : Group I (healthy), Group II (negative control) given DMBA 20 mg/kgBW twice weekly for five weeks orally, and Group III, IV and V (treatment groups) given DMBA and extract at the doses of 50, 100, and 200 mg/kgBW respectively everyday for 5 weeks orally. After week 16^{th}, the p53 and Bcl-2 gene expression was observed using immunohistochemistry. The average of expression of p53 gene at the extract doses of 50, 100, and 200 mg/kgBW were 3.05, 4.95, and 6.38% respectively. The doses of 100 and 200 mg/kgBW showed a significant difference ($p > 0.05$) from the negative control (2.53%). While the percentage of Bcl-2 expression were 10.22, 9.15, and 7.55% respectively which is not significantly different from negative control (11%). It can be concluded the ethanolic extract of *E. scaber* leaves increased the p53 gene expression at the dose of 100 and 200 mg/kgBW in rats breast cancer, but it can't decrease the expression gen of Bcl-2.

Keyword : *Elephantopus scaber* L, Breast Cancer, p53 , Bcl-2, Gene Expression

INTRODUCTION

Breast cancer is a malignant cancer that originates from glandular cells, gland ducts and breast support tissues, which are mostly related to hormonal and genetic factors (1). Center of Disease Control and Prevention reported breast cancer is the most incidence among all of the cancer deseases.

It is a type of cancer that causes the greatest death in women (2). Breast cancer occurs due to abnormal cells in the glands of the ducts and breast tissue that can invade other tissues of body (3). One gene that can control abnormal cell growth in cancer is the tumor suppressor gene p53 through interaction with the Bcl-2 family genes. The Bcl-2 gene works in contrast to p53 which disrupts the balance of cell cycle regulation. Cancer cells will proliferate and resistance to stimulation which normally results in cell death (4). The p53 expression is responsible to many kinds of tumor suppressor mechanism such as apoptosis, angiogenesis inhibition and cell cycle arrest (5,6). Apoptosis is a programmed cell death. Disruption of apoptosis process can increase tumor development. In apoptotic pathway, the p53 interacts with Bcl-2 which also controls cell death as anti apoptosis (6,7).

Cancer cell growth can be induced by carcinogen. One of the ingredients that can induce cancer is DMBA. The DMBA causes the proliferation of cancer cells through epoxide compounds formation by damaging DNA so that it can interfere the cell cycle. It also and cause the expression of p53 does not occur but the expression of Bcl-2 is excessive so that apoptosis does not occur (8). The two genes are often used to explore an anticancer agent from many sources.

One of the potencial anticancer source is the *E. scaber* leaf. It has been reported reported that it can prevent cell proliferation (9) and the active content of *E. scaber* is deoxyelephantopin. Deoxyelephantopin shows that the antiproliferative properties can be used to reduce the expansion of cancer cell proliferation by inhibiting DNA synthesis (9,10) Deoxyelephantopin also reveals a high cytotoxicity by inhibiting the growth of cancer cells by killing or damaging cells (9). Nevertheles, the anticancer molecular mechanisme of *E. scaber* leaves especially through the p53 and Bcl-2 genes has not been reported. Therefore, this study was carried out to determine the effect of the leaf extract of *E. scaber* as anticancer in rat. The anticancer assay was conducted by observing the expression of p53 and Bcl-2 genes in breast cancer of the female Sprague Dawley rats induced by DMBA using immunohistochemistry methods.

METHOD

Materials

The chemical ingredients used were *E. scaber* leaf obtained from Sentolo Kulon Progo, Yogyakarta, 70% ethanol, DMBA (7,12-dimethylbenz [α] anthrasen), tween 80, corn oil, and aquadest, and p53 and Bcl-2 antibodies.

Procedure

Extraction

E. scaber leaves were washed with water then dried using an oven at 600^{o}C. The dried *E. scaber* leaves are made of coarse powder and then macerated by 250 g of coarse powder in 1 L of 70% ethanol. The macerate obtained is then evaporated with a rotary evaporator to obtain a condensed extract.

Preparation of test solution (DMBA in corn oil)

DMBA induction is carried out at a dose of 20 mg/kgBW. The DMBA concentration is 0.4% in corn oil. The administration of DMBA for each mouse was done twice a week for five weeks orally.

Preparation of test solution (ethanol extract of *E. scaber* leaves in tween 80 5%)

The 2.5 g of Tween 80 were put into a 50 mL volumetric flask then the aquadest was added until the volume reach 50 mL, then shaken until it was dissolved and homogeneous. Ethanol extract of *E. scaber* leaves according to the dosage was then suspended in tween 80 5% solution. Test solutions are always made new before administration to test animals.

Distribution of sample groups

Sprague Dawley female rats aged 1 month were divided into 5 groups. Group I is a healthy control group. Group II was negative control given 20 mg/kgBW of DMBA orally 2 times a week for 5 weeks and extract solvent every day for 5 weeks. Groups III, IV, and V were given groups of ethanol extracts of *E. scaber* leaves with doses of 50, 100, and 200 mg/kgBW respectively every day for 5 weeks then given a break for 11 weeks to observe the appearance of nodules.

Immunohistochemistry test

The tissue in paraffin block was rehydrated using 100% ethanol, 95% and 70% for 2 minutes, 2 minutes and 1 minute respectively, and finally with water for one minute. The preparations was soaked in peroxidase blocking solution at room temperature for 10 minutes, then incubated in serum prediluted blocking at 25˚C for 10 minutes. After that, it was soaked in monoclonal anticaspase-3 antibody and Bax at 25˚C for 10 minutes, then washed using secondary antibodies (conjugated to horse radish) at 25˚C for 10 minutes, then PBS for 5 minutes. Futhermore, it was incubated with a peroxidase of 25˚C for 10 minute. Susequently, the preparation was washed with PBS for 10 minutes, then incubated with chromogen DAB (3,3-diaminobenzinidine) at 25˚C for 10 minutes and continued incubation with Hematoxyline Eosin for 3 minutes. Preparations are washed with running water, cleaned and dripped with mounting media, then covered with coverslip. Expression of p53 and Bcl-2 was observed using a light microscope with a magnification of 40x. Observation of p53 and Bcl-2 gene expression was carried out using a light microscope. Cells that express p53 and Bcl-2 will give a brown color while those that do not express p53 and Bcl-2 will be blue or purple. The percentage of cells expressing the genes was then calculated.

RESULTS

p53

The results of observations of expression in p53 are shown in Figure 1. In Figure 1 (A), the group healthy control looks a lot of blue tissues, but there are some brown tissues. This means that cells do not show p53 expression or apoptosis does not occur. Apoptosis did not occur because the

group was not treated with ethanol extract of *E. scaber* leaf. Some brown tissues were detected because in normal conditions cells in the tissues also undergo apoptosis (11). Figure 1 (B) is a negative control group induced by DMBA. The results in this group were mostly blue tissue and some were brown but the number was smaller than the normal control group. Apoptosis in this group does not occur because of the presence of DMBA which can cause mutations in apoptotic inducing genes such as p53. In Figures 1 (C), (D) and (E) are the groups that received *E. scaber* leaf extract treatment with doses of 50, 100 and 200 mg / kgBW respectively. The results in the group showed mostly brown. This indicates that most cells in the tissue undergo apoptosis, due to the administration of ethanol extract of *E. scaber* leaves.

DMBA-induced test animals express mutant p53 because DMBA reactive metabolites will form DNA adducts and cause mutations *(12)*. The mechanism of DMBA on the expression of p53 causes cell proliferation. Furthermore, the p53 gene will be activated and cause expression of p53 does not occur so that the cell cycle cannot stop at the end of the G1 phase and DNA repair will not occur *(8)*.

A previous study reported that the ethanol extract of *E. scaber* leaf can increase the expression of p53 and trigger cell death by apoptosis *(13)*. Deoxyelpehantopin can induce a cell cycle inhibition process in the G2/M phase and induce apoptosis in cancer cells *(10)*. Deoxyelephantopin of *E. scaber* leaf can be used as an anti-cancer by preventing the increase of proliferation of cancer cells by means of repairs to DNA that has mutations *(14)*.

The percentage analysis of p53 expression is done by counting the number of cells expressed divided by the total number of cells multiplied by 100%. Percentage of p53 expression in the control and treatment groups was presented in Figure 2 and Table I. The results in the healthy control group showed p53 expression of 2.70%, whereas in the negative control group was 2.53%. The percentage of both groups did not have a significant difference after statistically analyzed. The percentage of p53 expression in the treatment group at 100 and 200 mg/kgBW when compared to the negative control

group have a significant difference. This shows that the administration of ethanol extract of *E. scaber* leaves can affect the increase of p53 expression.

Bcl-2

The observation result of Bcl-2 expression is shown in Figure 3. Figure 3 (A) is that the healthy control group appears blue tissue, but there are some brown tissues. This means that cells show Bcl-2 expression or no apoptosis occurs. Apoptosis did not occur because the group was not treated with ethanol extract of *E. scaber* leaf. Some brown tissues are caused because in normal conditions cells in the tissues also experience apoptosis (11). Figure 3 (B) is a negative control group induced by DMBA. The results for this group are mostly blue tissue and some are brown but fewer in number than healthy control groups. Apoptosis in this group does not occur because of the presence of DMBA which can cause mutations in the antiapoptotic protein Bcl-2. In addition, the group was not given ethanol extract of *E. scaber* leaf which can reduce the expression of antiapoptotic protein Bcl-2. %. This shows that Bcl-2 expression is excessive due to the administration of DMBA as a carcinogen compound. Excessive expression of Bcl-2 causes uncontrolled abnormal cell growth so that the formation of cancer cells is difficult to inhibit. This shows that DMBA-induced test animals express large amounts of the Bcl-2 gene because the DMBA reactive metabolite, the epoxide compound, will form the DNA adduct and cause mutations. P53 mutations can cause an increase in Bcl-2 expression. Bcl-2 gene functions to protect tumor cells from the apoptosis process that allows cells to grow (8).

Figure 3 (C), (D) and (E) are the groups that received the treatment of ethanol extract of *E. scaber* leaves with a dose of 50, 100 and 200 mg/kgBW, respectively. The results for the group are mostly brown. This indicates that most cells in the tissue undergo apoptosis, due to the administration of ethanol extract of *E. scaber* leaves. This shows that the administration of ethanol extract of *E. scaber* leaf and DMBA can reduce the expression of Bcl-2, because the deoxyelephantopin content of the *E. scaber* leaves can be used as an anti-cancer by preventing the increase of cancer cell proliferation (14). Deoxyelephantopin can cause DNA damage by inhibiting DNA synthesis in cancer cells (9). The percentage analysis of Bcl-2 expression is done by calculating the number of cells expressed divided

by the total number of cells multiplied by 100%. The calculation results of% Bcl-2 expression obtained in each treatment group can be seen in Figure 4 and Table II.

The results in the healthy control group showed a Bcl-2 expression of 5.18%, whereas in the negative control group it was 11%. The percentage of both groups did not have a significant difference after statistically analyzed. So it can be concluded that the administration of DMBA does not specifically affect the expression of Bcl-2 but can cause nodule formation. The percentage of Bcl-2 expression in the treatment group at 50, 100 and 200 mg/kgBW when compared with the negative control group did not have a significant difference. This shows that administration of ethanol extract of *E. scaber* leaves cannot affect the decrease in expression of Bcl-2.

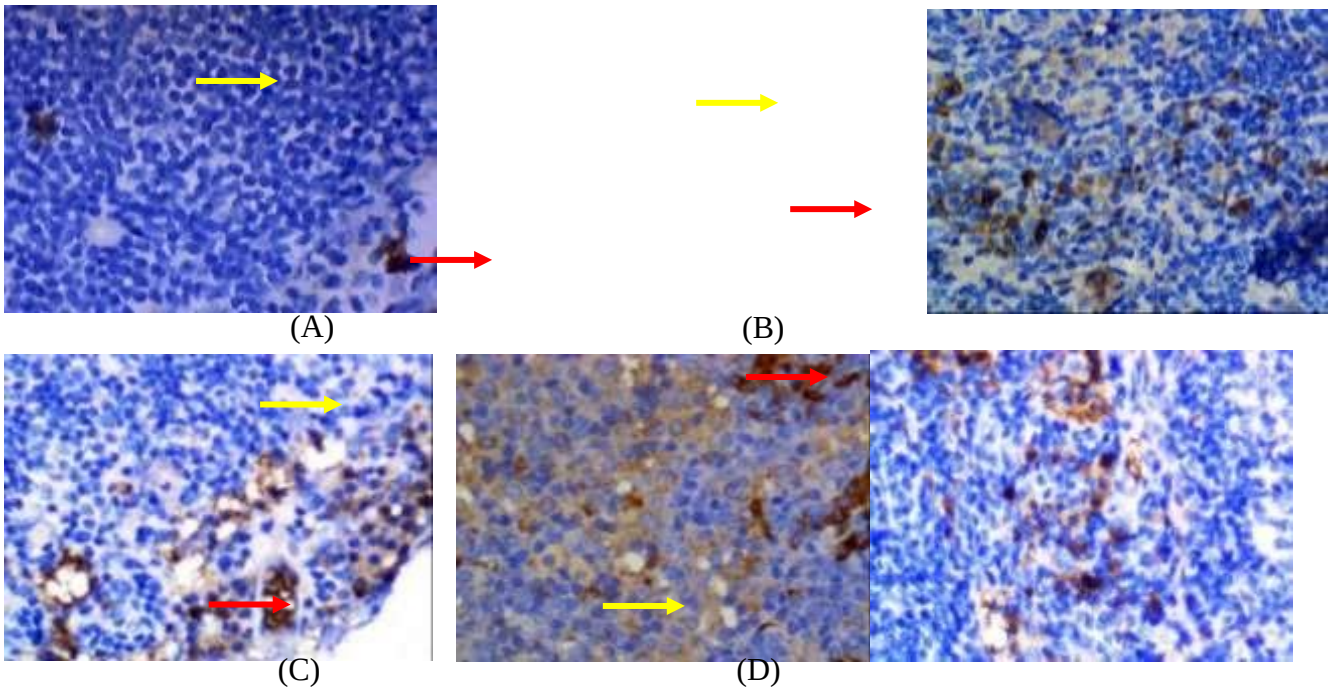

Figure 1. Microscopic photos of immunohistochemistry results p53 enlargement of 40x. Healthy control (A); DMBA group 20 mg / kg BW (B); extract *E. scaber* 50 mg / kg BW (C); extract *E. scaber* 100 mg / kg BW (D); *E. scaber* extract 200 mg / kgBW. Cells not expressing p53 (blue) is pointed by yellow arrow; Cells expressing p53 (brown color) is pointed by red arrow.

(E)

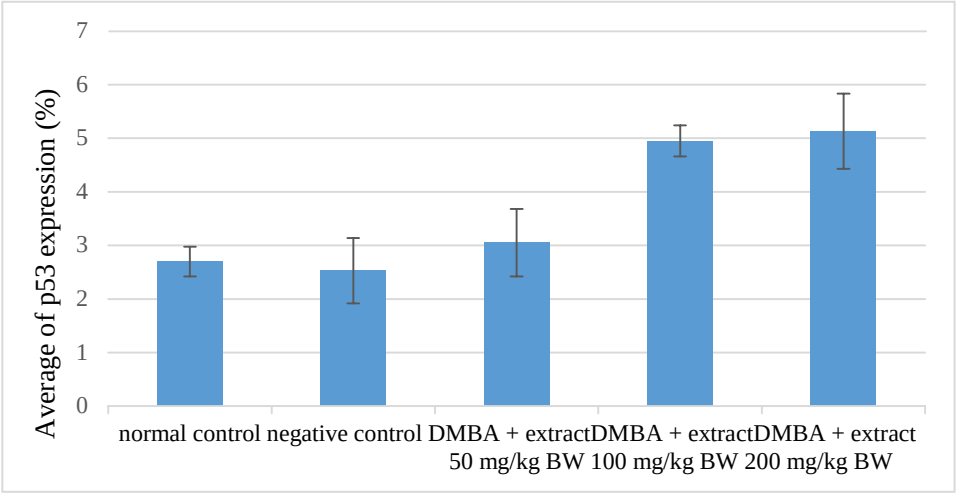

Figure 2. Percentage of p53 expression in the control and treatment groups

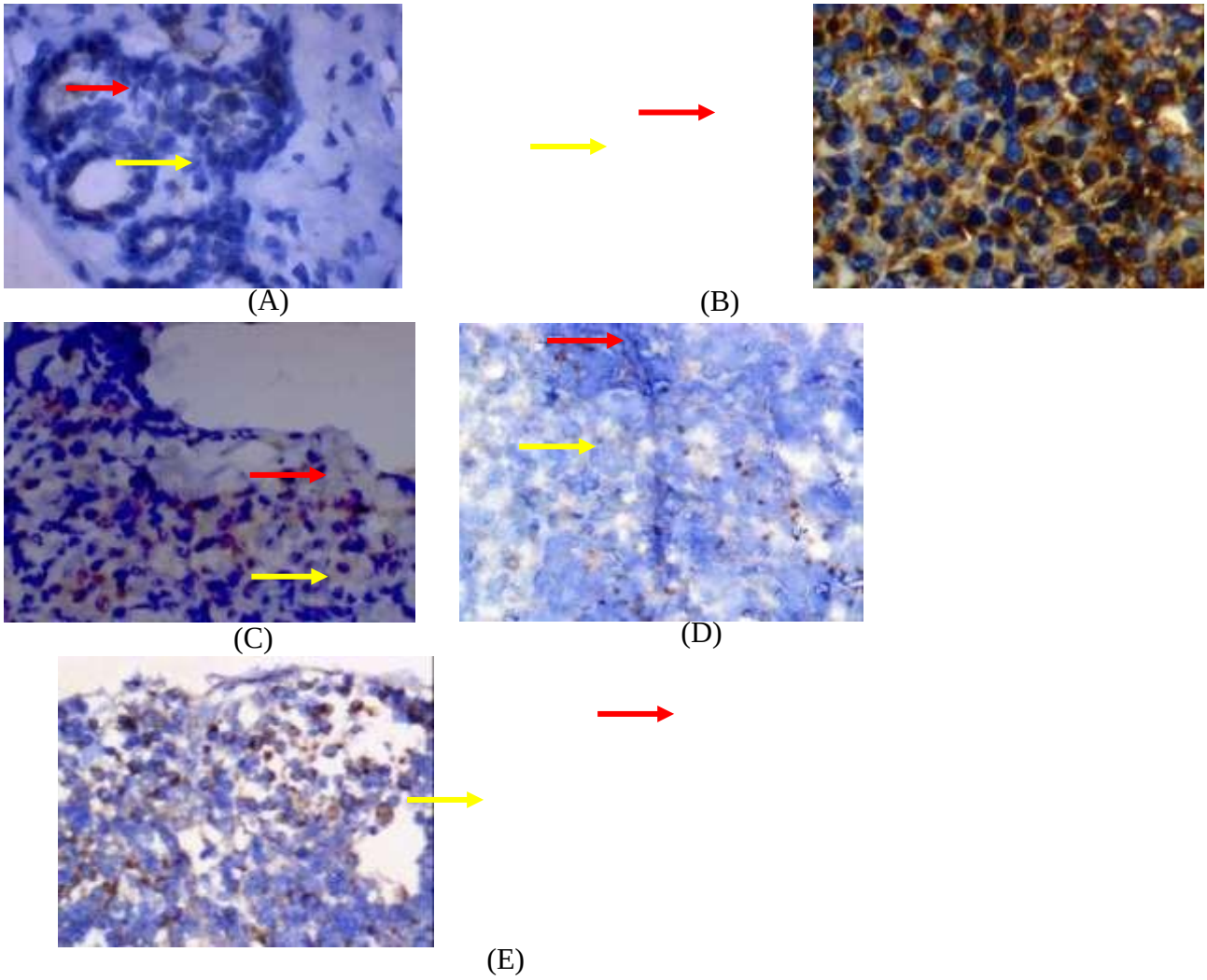

Figure 3. Microscopic photos of immunohistochemistry results of Bcl-2 40x magnification. Healthy control (A); DMBA group 20 mg/kgBW (B); extract E. scaber 50 mg/kgBW (C); extract E. scaber 100 mg/kgBW (D); E. scaber extract 200 mg/kgBW. Cells not expressing Bcl-2 (blue) is pointed by yellow arrow. Cells expressing Bcl-2 (brown) is pointed by red arrow

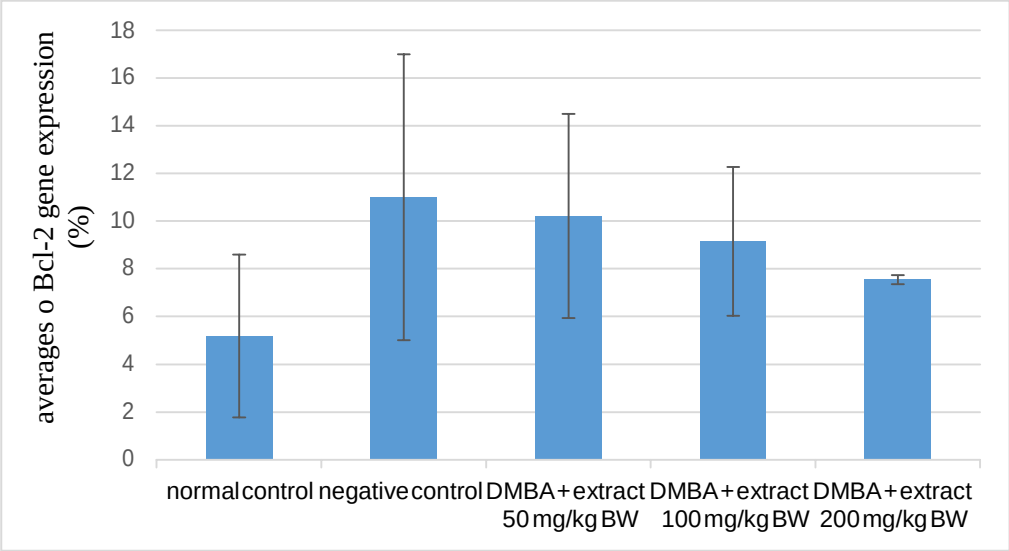

Figure 4. Percentages of Bcl-2 expression in the treatment of healthy groups, negative control, extract group 50 mg/kgBW, extract group 100 mg/kgBW, and extract group 200 mg/kgBW

Table I. Percentage of expression of p53 in each group by giving ethanol extract of *E. scaber* leaf

Groups	**Average of p53 Expression (% ± SD)**
Normal control	2.73 ± 0.28
Negative control	2.53 ± 0.61
DMBA + extract 50 mg/kgBW	3.05 ± 0.63
DMBA + extract 100 mg/kgBW	4.95 ± 0.29
DMBA + extract 200 mg/kgBW	6.38 ± 0.70

Table II. Percentage of Bcl-2 expression in each group by giving ethanol extract of *E. scaber* leaf

Groups	**Averages of Bcl-2 Expression (% ± SD)**
Normal control	5.18 ± 3.41
Negative control	11 ± 5.99
DMBA + extract 50 mg/kg BW	10.22 ± 4.28
DMBA + extract 100 mg/kg BW	9.15 ± 3.12

DMBA + extract 200 mg/kg BW	7.55 ± 0.19

CONCLUSIONS

The ethanol extract of *E. scaber* leaf significantly increased p53 gene expression at the dose of 100 and 200 mg/kgBW in Sprague Dawley female rats induced by DMBA. The dose of 50, 100 and 200 mg/kgBW of the extract decreased Bcl-2 gene expression in the rats but they were not significanly different.

ACKNOWLEDGEMENT

I would like to thank to Ministry of Research,Technology and Higher Education of the Republic of Indonesia for giving research grant through the Hibah Penelitian Strategis Nasional Institusi.

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Elephantopus scaber Linn Extract Induces Apoptosis and Activates Caspase Cascade in T47D Cancer Cell Line

Abstract

Elephantopus scaber Linn. was used traditionally for treating various diseases. Previous studies found the cytotoxicity of this herb against different cell lines. This study aimed to determine the effectiveness of the ethanolic extract of *E. scaber* in inducing apoptosis by observing its effect on caspase cascade. This ethanolic extract was obtained by maceration method using 96% ethanol. It was fractionated with petroleum ether to discard compounds with very low polarity. This step was followed by the fractionation with chloroform to isolate compounds with optimum polarity. The soluble part in chloroform was dried and used for the assay. The immunocytochemistry method used specific antibodies for caspase-8, caspase-3, and caspase-9 to observe the expressions of each caspase. T47D cell line was treated with the chloroform fraction of *E. scaber* with the concentrations of 7.06 µg/ml and 3.53µg/ml. The results of the immunocytochemistry showed that this chloroform fraction increased the expressions of caspase-8, caspase-3, and caspase-9 proteins significantly. Such increase led to apoptotic cells. This finding supported the development of *E. scaber* extract as an anticancer agent.

Keywords: *Elephantopus scaber*, apoptosis, caspase-8, caspase-3, caspase-9, immunocytochemistry

1. Introduction

Cancer is a major health problem that causes death after cardiovascular disease. Breast cancer is the first ranked most common malignancy in female population [1]. Although cancer treatment has currently used different methods, it still does not provide any satisfying results. Also, cancer therapy has many side effects and damages the normal cells.

Cancer progresses due to an imbalance between cell proliferation and cell death. The process of programmed cell death, or apoptosis, is considered vital in normal homeostatic settings. This process produces a balance in the number of cells by eliminating damaged cells and physiological proliferation. The defects in the mechanisms of apoptosis play essential roles in tumor development as they allow neoplastic cells to survive and reproduce uncontrollably[2].

Elephantopus scaber L. has been used traditionally to treat various disease. It has been reported to have a cytotoxic effect and induce the apoptotic death of HeLa cancer cells [5]. Some of its active compounds have been successfully isolated, particularly deoxyelephantopin and isodeoxyelephantopin of the sesquiterpene lactone class [3]. Deoxyelephantopin inhibits the growth of cancer cells by an

apoptotic mechanism in which the caspase cascade, i.e., caspases-8, -9, -3, and -7, are activated [4]. This finding affirms the potential of *E. scaber* L.as an anticancer [7].

The fractions obtained from the extract of *E. scaber* L. are empirically found to exhibit cytotoxic activities, which are indicated by IC_{50} value. The chloroform fraction of the leaves of *E. scaber* L. shows cytotoxic activity against T47D breast cancer cell line with IC_{50} of 7.06 ug/ml [6]. The understanding of the mechanism by which a compound works is fundamental to drug development. This study aimed to identify the efficacy of the active fraction of the ethanolic extract of *E. scaber* L. in inducing apoptosis by the activation of caspase cascade.

2. Materials and Methods

2.1. Materials

The leaves of *E. scaber* L. were obtained in Yogyakarta, Indonesia. The plant was identified as *Elephantopus scaber* Linn. from the family Compositae in Laboratory of Biology, Universitas Ahmad Dahlan under the supervision of Assoc. Prof. Hadi Sasongko, and the number of the herbarium specimen is 073/Lab.Bio/B/VII/2016.

2.2. Extraction and Fractionation

The leaves of *E. scaber* L. were dried in an oven at 50^{o}C. The dried leaves were powdered and sieved with 20/40 mesh. The powder that passed through the 20 mesh but was retained by the 40 mesh was used for extraction. The extraction was performed by maceration with 96% ethanol solvent. The maceration was replicated three times to maximize the collection of the compounds. The macerate was evaporated with a vacuum evaporator to obtain a viscous extract.

The viscous extract of 20 grams of *E. scaber* L. was dissolved and shaken in 100 ml of petroleum ether to discard compounds with very low polarity. The soluble fraction of the petroleum ether was separated from the insoluble matter, which was later dissolved in 100 ml of chloroform. The chloroform fraction was evaporated until a solid chloroform fraction was formed. The fractionation of the extract with chloroform was performed three times to get the maximum active fraction.

2.3. Sample Preparation

A sample of 10 mg was dissolved in 1 ml of dimethyl sulfoxide (DMSO). Then, it was diluted with RPMI to achieve the concentrations of 7.06 μg/ml and 3.53 μg/ml by gradual dilution. The treatment used these two concentrations because the IC_{50} of chloroform fraction was 7.06 ug/ml [8]. At this concentration, the cell growth and protein expression were easily observable due to the presence of

adequate viable cells. The final concentration of DMSO in the sample was 0.007%. Exposure to 1% DMSO is empirically proven to inhibit cell survival insignificantly [9]. Therefore, this concentration is not toxic to cell growth.

2.4. Immunocytochemistry

The expressions of the caspases were observed with immunocytochemistry technique [10]. T47D cells were grown in a 24-well microplate. They were left to attach and grow in the bottom of the plate after overnight incubation. The microplate was taken from the incubator, and the culture medium was then removed from each well using a micropipette. A solution of 1 ml were sampled from extract with concentrations of 7.06 µg/ml and 3.53 µg/ml, transferred into the well, and then incubated for 24 hours. After the incubation, all culture mediums were removed from the well, added with 300 µl of PBS, and then left for 5 minutes. The PBS solution was discarded. The culture mediums were added with 300 µl of distilled water, left for 5 minutes, and then discarded. The cells were fixed with 300 µl of methanol and left for 10 minutes before the methanol was discarded. After the fixation, the cells were washed two times with 300 µl PBS, added with 100 µl of hydrogen peroxide solution, and left for 5-10 minutes. The solution was removed and washed with 300 µl of PBS two times. Afterward, the cells were added with 100 µl of prediluted blocking serum and left for 10-15 minutes. They were then removed, added with 100 µl of primary anti-caspase-8, anti-caspase-3, and anti-caspase-9, and incubated for 24 hours. After the incubation, they were washed two times using 300 µl of PBS, added with 100 µl of secondary antibodies, and left for 20 minutes. Afterward, the cells were washed two times using 300 µl of PBS, added with 100 µl of HRP solution, left for 10 minutes, and then washed with PBS.

DAB solution were added to the cells and left for 2 minutes. After washing them with distilled water, the microplate was added with Mayer Hematoxylin solution and left for 5 minutes. The last step was washing the cells with 500 µl of distilled water and left them to dry. The expressions of caspase-8, caspase-3, and caspase-9 were observed under a light microscope.

2.5. Analysis

Cells were observed under a light microscope with 100x magnification. The expressions of caspase 8, caspase-3, and caspase-9 were characterized by the color of the cell. The positive caspases appear in brown or dark color, while the negative ones have blue or purple color. The expressions were observed on six fields of view for every sample and presented as the percentage of positive expression compared to the total area of the cells.

3. Results and Discussion

3.1. Extraction and Fractionation

The extraction of the leaves of *E. scaber* L. using 96% ethanol produced a concentrated extract with a dark color. The yield of the extraction was 8.5%, which was in line with the standard, i.e., higher than 2.7% [11]. Fat and other compounds with very low polarity were then removed with petroleum ether. Fractionation with chloroform aimed to isolate active compounds with optimum polarity. The result was 20.5% compared to the crude extract. The high cytotoxicity of chloroform fraction was indicated by IC_{50} of 7.06 µg/ml [6].

3.2. The Increased Expression of Caspase-8 by the Elephantopus scaber Extract

The activation of apoptosis-signaling pathways by anticancer drugs is frequently formed during the activation of caspases, a family of cysteine proteases that act as common death-effector molecules. Caspases can trigger apoptosis by cleaving various cytoplasmic or nuclear substrates, which are the morphologic features of apoptotic cells. The activation of caspase can be initiated in the plasma membrane with different mechanisms, by either death receptor-mediated signaling (receptor pathway) or mitochondrial pathway [12].

Elephantopus scaber was reported as a promising anticancer agent. Some active compounds that are isolated from *E. scaber* exhibit cytotoxic activity against some cell lines [4,13]. This study observed the expression of caspases after the addition of the fraction of *E. scaber* as a potential treatment for cancer. The expression of caspases involved a series of complex processes and many factors. The gene expression system, including initiation, transcription, translation, and other concomitant processes, was carefully controlled.

The expression of caspase-8 in T47D breast cancer cell line after treatment with *E. scaber* extract is presented in Figure 1. Treatment with 3.53 µg/ml increased the expression of caspase-8 (Figure 1B). The dark brown cells were identified in nearly all culture cells. This expression was significantly different from the morphology of the control sample (Figure 1A). The dark brown color indicates high expression of caspase-8 following the treatment with *E. scaber*. A higher dose of the fraction of *E. scaber* (7.06 µg/ml) resulted in damaged cells (Figure 1C). Subsequently, the T47D cells entered the late stage of apoptosis and, then, necrosis. The morphology of the T47D cells was characterized by cellular shrinkage and apoptotic bodies in their surrounding. The morphological changes, signifying apoptosis, were observed in most cell types. These changes started with a reduction in cell volume and followed by the condensation of the nucleus [14].

At a concentration of 3.53 µg/ml (Figure 1B), treatment with *E. scaber* produced brown cells, indicating the expression of caspase-8 in the cytoplasm. This expression led to apoptotic cells. The calculation of the expression is summarized in Table 1.

The activation of caspase-8 after the treatment indicates the activation of the extrinsic pathway. Caspase-8 also has a significant role in the transcription of p53 tumor suppressor protein [15].

Figure 1. The expressions of caspase-8 in T47D cells after treatment with *E. scaber*: (A) Control cells, (B) 3.53 ug/ml, and (C) 7.06 ug/ml.

Table 1. The calculation of the expression of caspase-8 in T47D cells after treatment with *Elephantopus scaber*

Treatments	The Expressions of Caspase-8 (% ± SD)
control	0 ± 0%
3.53 µg/ml	96.62 ± 2.69%
7.06 µg/ml	100 ± 0%

3.3. The Increased Expression of Caspase-9

Caspase-9 is an initiator caspase that regulates the occurrence of apoptotic processes through the internal pathway. It is activated by binding cytochrome c to Apaf-1, which forms a complex known as apoptosome. This complex activates the caspase-9 zymogen (pro-caspase-9). Once activated, caspase-9 will trigger the activation of the effector caspase and cause apoptosis [16].

This research showed that the treatment of T47D cells with *E.scaber* increased the expression of caspase-9 (Figure 2). The calculation of the expression of caspase-9 after the treatment is presented in Table 2.

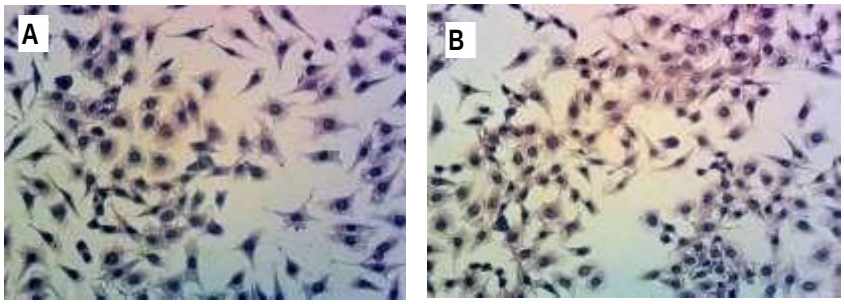

Figure 2. The expression of caspase-9 after treatment with *Elephantopus scaber*: (A) control and (B) 3.53 µg/ml

Table 2. The calculation of the expression of caspase-9 in T47D cells after treatment with *Elephantopus scaber*

Treatments	The Expressions of Caspase-9 (% ± SD)
Control	18.89 % ± 0.03
3.53 µg/ml	45.23 % ± 0.02

This study found that the expression of caspase-9 significantly increased after treatment with 3.53 µg/ml of *E. scaber* extract. In a normal condition (the control sample), the expression of caspase-9 in T47D cell lines was low (Figure 2A). After the treatment, it started to increase, proving the role of *E. scaber* in inducing of apoptosis through mitochondrial pathway.

3.4. The Increased Expression of Caspase-3

Caspase-3 is a proapoptotic agent that acts as a major effector caspase (executioner) in the process of apoptosis. It plays an essential role in breaking the apoptotic substrate and activating other effector caspases, including caspase-6 and caspase-7 [17]. Treatment with *E. scaber* extract was able to increase the expression of caspase-3, as presented in Figure 3 and Table 3.

During the research, caspase-3 seemed to be downstream of caspase-8. The increased expression of caspase-3 after treatment with *E.scaber* was most likely caused by the increased expression of caspase-8. Caspase-8 is an initiator caspase that cleaves pro-caspase-3 into activated caspase-3. Upon the activation, caspase-3 becomes capable of cleaving many cellular substrates and induces morphological changes like chromatin condensation, membrane blebbing and DNA fragmentation, indicating the process of apoptosis [18].

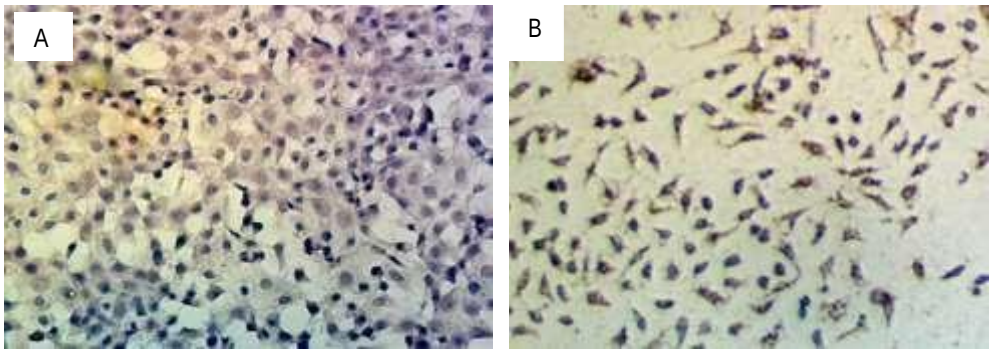

Figure 3. The expression of caspase-3 after treatment with *Elephantopus scaber*:

(A) control and (B) 3.53 µg/ml

Tabel 3. The expression of caspase-3 in T47D cells after treatment with the extract of *Elepahantopus scaber*

Treatments	The expressions of caspase-3 (% ± SD)
Control	2.13 ± 0.028
7.06 µg/ml	17.65 ± 0.018

3.5. Elephantopus scaber-induced caspase cascade

The activation of caspase-3 involved the intrinsic and extrinsic pathways. Caspase-3 was activated via the extrinsic pathway (death ligand) where the death signal caused by the compound of *E. scaber* bound to the death receptor. This bond formed a trimer with FADD (Fas-Associated Death Domain) and activated pro-caspase-8. The active caspase-8 activated caspase-3 as an effector caspase.

Meanwhile, in the intrinsic pathway (mitochondria), treatment with E. *scaber* extract induced the release of cytochrome c, which later formed a complex with Apaf-1 and pro-caspase-9 known as apoptosome. The active caspase-9 activated caspase-3 as an effector caspase. This study found that after the treatment with *E. scaber* extract, the expressions of caspase-8, caspase-9, and caspase-3 were increased, suggesting that this extract induces the apoptosis through intrinsic and extrinsic pathways.

The results of this study were in line with previous research, which reported that caspase-3 induced apoptosis and mediated cell cycle arrest in T47D cells by isodeoxyelephantopin. *E. scaber* was also reported to induce cell cycle arrest at G2/M phase [4].

This study proved the potential of *E. scaber* as an anticancer agent. The extract of *E. scaber* exhibits cytotoxicity against various cancer cell lines, including MCF-7 breast cancer cell lines [4,13], A549 lung carcinoma cells [4], Hela cervical cancer cell lines [19], HCT human colon cancer cell lines, and Daltons Lymphoma Ascites (DLA) tumor cells [3].

This study also confirmed that the mode of death induced by *E. scaber* was apoptosis. The ability to induce apoptosis is an essential requisite of anticancer agents, including chemotherapeutic agents, hormones, and various biological compounds [16]. This study found that *E. scaber* induced the apoptosis of T47D breast cancer cell lines by activating caspase cascade. The expression of caspase-9, caspase-8, and caspase-3 increased significantly. Therefore, *E. scaber* is potentially developed as an anticancer agent.

Acknowledgment

This research was supported by the Indonesian Ministry of Research, Technology, and Higher Education as a part of the Fundamental Research Scheme.

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